

**Alterations in the apoptotic:mitotic ratio : an early
indicator of breast cancer sensitivity to tamoxifen?**

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I confirm that this thesis has been composed by myself.

I acknowledge that the work reported was done by myself, with the exception of the assessment of bcl-2 staining in the clinical tumours, performed by Dr. A. Hanby of the ICRF, London. I am however indebted to several people for advice, instruction and assistance in the preparation of materials, as acknowledged on page 189.

Contents

| | page |
|---|---------|
| Abstract | 2 |
| Introduction | 3 |
| Aetiology | 5 |
| The normal breast | 7 |
| Hormonal treatment of breast cancer | 9 |
| Aims of this study | 15 |
| Potential markers for tamoxifen response | 16 |
| In vitro and in vivo effects of tamoxifen | 35 |
| Assessment of response | 39 |
| Methods & Materials | 45 |
| Study design | 45 |
| Materials | 46 |
| List of reagents | 55 |
| Methods | 58 |
| Results (contents in detail) | 76 |
| Methods | 78 |
| Clinical tumours | 84 |
| ZR-75-1 xenografts | 110 |
| MDA-MB-231 xenografts | 130 |
| Synchronously grown ZR-75-1 and MDA-MB-231 Xenografts | 141 |
| Sequential Fine Needle Aspirates of ZR-75-1 and MDA-MB-231 Xenografts | 148 |
| Analysis of individual ZR-75-1 and MDA-MB-231 xenografts | 159 |
| Discussion | 183 |
| Conclusion | 187 |
| Acknowledgements | 189 |
| Bibliography | 190 |

Abstract

Anti-oestrogens, such as tamoxifen, are widely used in the treatment of breast cancer, although a significant minority fail to respond. Apart from tumour expression of Oestrogen Receptors (ER), there are no other good pre-treatment predictors of sensitivity. The management of patients would be facilitated by early prediction of efficacy as current practice requires a trial of several weeks' therapy. In the clinical setting the precise effects of tamoxifen on tumour cell turnover remain unclear, but in model systems a significant anti-proliferative effect has been demonstrated, as well as a possible induction of apoptosis. Their relative balance, the apoptotic:mitotic ratio, might therefore be a predictor of drug sensitivity. The use of the Gompertz function, an accurate model of xenograft growth, could be used to predict subsequent tumour behaviour and thus identify responsive disease. The aim of this study was therefore to determine the relationship between tumour response to tamoxifen and changes in apoptosis and mitosis, to confirm their chronology and specificity in a model system, and to assess the possibility of identifying these changes in fine needle aspirates (FNAs) rather than biopsy material.

In a cohort of 50 post-menopausal women with ER positive breast cancer treated with tamoxifen, the level of expression of both ER and the cell-survival gene bcl-2 predicted for tumour response. After three months' treatment there was a significant fall in proliferation in most tumours, but no overall change in apoptosis, as assessed by morphology. There was a significant trend for tumour response to correlate with the change in the apoptotic:mitotic ratio ($p < 0.05$). To determine more accurately the timing of these changes, mice bearing xenografts of the ER positive ZR-75-1 and ER negative MDA-MB-231 breast cancer cell lines were treated with tamoxifen. After 2 days, an increase in apoptosis and the apoptotic:mitotic ratio was seen in the sensitive ZR-75-1 tumours ($p < 0.05$), and on day 7 there was also evidence for reduced proliferation ($p < 0.05$); however clear evidence of tumour regression only occurred after 14 days. On day 7 there was however a significant correlation between the apoptotic:mitotic ratio and the difference between the actual and the Gompertz-predicted tumour volumes ($p < 0.02$). These changes were not apparent in tamoxifen treated MDA-MB-231 tumours. No changes in bcl-2 expression were seen. Furthermore, although similar changes were seen in tumour FNAs, statistically they were not as significant, suggesting that they do not offer an alternative to repeat histological examination of the tumours.

Early changes in the apoptotic:mitotic ratio during tamoxifen treatment of breast cancer may aid in the prediction of endocrine sensitivity.

Introduction

Breast Cancer is the commonest cancer to affect women in Scotland (Sharp et al(1993)), as it is throughout the developed world. Almost a quarter of all female cancer registrations are for breast cancer (Sharp et al(1993)), and it is estimated that an American woman has a life-time risk of about 1 in 8 for developing this disease (Feuer et al(1993)). However it needs to be emphasized that a third of this risk is after the age of 75 (Henderson, (1990)), and perhaps a more useful statistic is the 1 in 15 risk quoted for Scottish women up to the age of 75 (Sharp et al(1993)). Breast Cancer thus presents a major health-care problem, although the overall 5-year survival for women in Scotland who develop this disease is 64% (Sharp et al(1993)). Furthermore there is evidence that the incidence of this disease is increasing, both in Scotland (Sharp et al(1993)) and elsewhere (Feuer et al(1993)), although the mortality appears unchanged. There are two factors that may explain this rise ; increased detection, which results in part from national screening programmes, and increased longevity, since the disease is more prevalent in older women. There is also a steady unexplained increase in incidence over the past fifty years that may be due to environmental or dietary changes. There is however world-wide variation in this increase, and indeed for Scotland the 12% increase seen during the decade 1980-1990 was no more than for all cancer registrations as a whole. However it needs to be remembered that the incidence of other tumours, such as gastric cancer, is clearly falling, and furthermore that the nationwide breast cancer screening did not begin until the end of that decade. Before discussing the aetiology of this disease, which will have relevance both to the rising incidence as well as the impact of therapy, it is important to review some recent data which offer hope in the face of this increasing problem.

For many years the mainstay of treatment for early, and thus potentially curable, breast cancer was surgery, sometimes with post-operative radiotherapy. However since the late 1940's there have been numerous trials conducted throughout the world designed to assess the survival benefit of the use of post-operative adjuvant systemic therapy. A recent overview of over 100 trials of chemotherapy, hormone therapy and immunotherapy confirmed that the survival of women with early breast cancer is improved by this approach (Early Breast Cancer Trialists Collaborative Group, (1992)). The benefit of such therapy for patients outwith the minority who were entered into these randomised trials has been confirmed by a retrospective analysis of cohorts of women with breast cancer treated in British Columbia (Olivotto et al(1994)), (which incidentally also confirmed the increased incidence of the disease). There was a significant improvement in survival for younger women between 1974 and 1980, which corresponded to the introduction of adjuvant chemotherapy for pre-menopausal women with node-positive disease. In contrast, there was no difference in survival for

post-menopausal women in the same period. Between 1980 and 1984 there was a significant improvement for both groups of women, corresponding to the widespread use of tamoxifen for post-menopausal women with node-positive or high-risk node-negative tumours, and the further use of chemotherapy for pre-menopausal women with high-risk node-negative disease. The authors thus argue that it is the use of adjuvant systemic therapy that has brought about this improvement ; they chose this third period not to include the large numbers of women whose breast cancer had been diagnosed after the advent of nationwide mammographic screening programmes. Similar retrospective analyses have also confirmed improved survival due to systemic adjuvant therapy in the U.S. (Chu et al(1996)) and England and Wales (Beral et al(1995)).

Aetiology

It has to be stated that, as with most other cancers, the reason an individual develops breast cancer remains unknown. As a common disease, it has been extensively studied, and several factors are known to increase the risk of developing breast cancer. In contrast much less is known about how women might reduce their risk, which confirms that some of the fundamental issues involved in the development of this malignancy remain unclear.

Genetic influences

There is no doubt that for some women at least, there is an increased risk of breast cancer related to the occurrence of the disease in other members of their family. It is well known that the risk of developing the disease can be two to five times higher if one or more first or second degree relatives have been affected (Anderson et al(1985)). These data alone do not confirm the possibility of a genetic predisposition, but there is evidence for families in which the women are at very high risk of developing breast cancer (Lynch et al(1984)), for whom an autosomally dominant gene, inheritable from either parent, is felt to be the major cause. Worldwide collaboration, based on over 200 such families that have been identified, has led to the sequencing of two genes, mutations in which are thought to be the cause of the majority of familial breast cancer ; BRCA-1 (Miki et al(1994)) and BRCA-2 (Wooster et al(1995)). To date the precise function of either gene remains undetermined, and there is no evidence that mutations in these genes are significantly involved in the development of sporadic breast cancer. There is also evidence that acquired genetic mutations may be important in the aetiology of breast cancer. Women whose breasts have been exposed to ionising (and thus mutagenic) radiation are at increased risk of breast cancer, irrespective of whether this was from fluoroscopic examination for tuberculosis (Boice et al(1977)), radiotherapy for post-partum mastitis (Shore et al(1986)), or Japanese women who survived the atomic bombs dropped on Hiroshima and Nagasaki (Tokunaga et al(1982)). Another significant risk factor for breast cancer is a previous diagnosis of certain forms of benign breast disease, most significant amongst which is atypical ductal hyperplasia, with a 3-5 times increased risk (Carter et al(1988); Dupont et al(1985)). Whether this is due to a genetic mutation that occurs during or after the benign hyperproliferative stage is unknown, but the incidence of cyclin D1 abnormalities was noted to be similar for in-situ and invasive carcinomas (Weinstat-Saslow et al(1995)), whereas they are much less common in benign hyperproliferative conditions, suggesting that mutation may occur during the transition to malignant disease.

Geographical influences

Genetics apart, there is also significant geographical variation in the incidence of this disease. The best publicised is the difference between the developed part of the Western World where the age-standardised incidence can be as high as 100/ 100,000 women, and Asian countries where it may be as low as 15/100,000. When women from a low-risk group move to a region with a higher incidence, their own risk increases, even though this may take more than a generation to occur (Wynder et al(1986); Ziegler et al(1993)). Furthermore the incidence is often higher in urban than rural areas, with some groups, such as Mormons, having a much lower incidence (Adami et al(1990)). The reason that living in the Western World itself appears to be a risk factor for breast cancer is uncertain, but the dietary intake of fat is often considered to be the key factor. However the strong associations between national dietary fat intake and incidence of breast cancer is not as well supported by studies in individuals, with inconsistent results being found in case-control studies (Adami et al(1990)). Of more significance are studies where a very low fat intake appears to reduce the incidence of the disease (Lubin et al(1986)), an effect potentially mediated through decreased levels of circulating oestradiol (Prentice et al(1990)).

Hormonal influences

When considering the factors associated with a small, but significant increase in the risk of developing breast cancer it becomes apparent that apart from familial associations, the majority are linked to the hormonal milieu within the woman. For example, the use of post-menopausal exogenous oestrogen, as in H.R.T., may increase the risk, particularly if administered for 5 or more years (Colditz et al(1995)). In contrast, early age at first birth may decrease the risk of a subsequent breast cancer (MacMahon et al(1970)), although re-evaluation of the data suggests that the age of subsequent births may also alter the risk (Trichopoulos et al(1983)). Equally prolonged use of the oral contraceptive may increase the risk of developing a cancer (Chilvers et al(1989)), although other large studies have not confirmed this (Stadel et al(1985)). However more definite is the decreased risk associated with an early menopause (Negri et al(1988)), which when induced by bilateral oophorectomy before the age of 40 is associated with only half the chance of subsequently developing breast cancer (Adami et al(1990)). In contrast, an early menarche or delayed menopause cause an increased risk by about 5% per year (Abeloff et al(1995)). These data all suggest that prolonged exposure to female sex-hormones, and oestrogen in particular, increase the risk of being diagnosed with breast cancer, but do not explain the mechanism for this link. Nonetheless, a recent study does however suggest a possible link between the genetic and non-genetic risk factors for the development of breast cancer - it seems that the expression of the BRCA-1 protein, at least in vitro, is under the control of oestrogen (Guda et al(1995)).

The normal breast

Some discussion of the normal breast and its hormonal control will be relevant to the understanding of the rôle of oestrogen in the development of breast cancer. The breast, which is fundamentally a secretory organ, has as its functional unit the lobule. This arises from a terminal duct and is composed of blind-ended ductules (acini) surrounded by a layer of luminal cells and then a further layer of myoepithelial cells. All these structures are in turn surrounded by a specialised mesenchyme, and the whole is referred to as the terminal duct lobular unit (TDLU). It is from the luminal epithelial cells of the TDLU that most pre-invasive and invasive breast cancers arise (Wellings et al(1975)). During the normal menstrual cycle, the luminal epithelium proliferates, with the maximum growth rate found during the mid-luteal phase, whether measured by ^3H thymidine labeling(LI) or mitotic index (MI) (Anderson et al(1982); Goings et al(1988); Potten et al(1988)). There was no significant variation in the degree of proliferation during the menstrual cycle with the use of the combined oral contraceptive (Anderson et al(1982); Potten et al(1988)), whereas there was a decrease with increasing age (Potten et al(1988)). The luteal phase of the menstrual cycle is associated with high levels of both oestrogen and progesterone, whereas during the follicular phase it is only the oestrogen levels that rise. Oestrogen exerts its effect on sensitive cells by binding to the Oestrogen receptor (ER), which in turn interacts with specific DNA regions, the oestrogen-response elements (ERE) (Green et al(1988)), which are in the vicinity of target genes whose transcription is thus modulated. Similarly, progesterone acts via the progesterone receptor (PgR), which itself is expressed under the influence of oestrogen (Horwitz et al(1975)). However there appears to be a negative-feedback control of the expression of ER within the normal breast, since the level of ER falls during the menstrual cycle, particularly in women on the combined oral contraceptive pill. These data point to a continuum of changes in cell proliferation and gene expression during the menstrual cycle, which can be considered as being necessary to prepare the breast for pregnancy should it occur. Hence a woman who has more years of regular menstruating without intervening pregnancy, such as would occur with earlier menarche, delayed menopause or a later first pregnancy (all factors which increase the lifetime risk of developing a breast cancer), will have exposed her luminal epithelial cells to more cell divisions. Since genetic mutations can occur at each mitosis, prolonged menstruation creates an environment in which there is a higher risk of transforming a breast epithelial cell into a malignancy.

Breast cancers usually retain some of the phenotype of their originating organ - few are so poorly differentiated as not to be identified as of breast origin. Concomitant with the retention of some of the hallmarks of breast epithelial cells is the expression of oestrogen receptors. Tumours presenting in post-menopausal women are more commonly ER-positive, probably due to increased levels of the

protein in cells that express ER (Gaskell et al(1992b)) (rather than an increased number of cells expressing ER). This is consistent with the observations in the normal breast, since post-menopausal women have much lower levels of circulating oestrogens and progesterone, and thus there is less stimulus to drive the negative-feedback loop that seems to be apparent in the luteal phase of the menstrual cycle. The expression of oestrogen receptors affords a mechanism where circulating, or locally synthesised oestrogen, can stimulate the proliferation of a breast cancer. Hence it is possible that prolonged exposure to oestrogens, which is associated with an increased risk of breast cancer, could stimulate the growth of a clinically silent tumour, and thus increase the chance of detection. Since most studies of risk factors for breast cancer do not assess the *life-time* risk for the women under study, but rather correlate the incidence in a cohort with various factors, it is possible that any apparent increased risk is not due to increased initiation of cancers, but earlier detection of oestrogen-sensitive malignant lesions stimulated by for example exogenous oestrogen in H.R.T. One would therefore anticipate that oestrogen receptors would more often be found in the breast cancers detected in women with a hormonally-related increased risk for the disease ; and positive associations between H.R.T. use and both higher ER concentration in the tumour (Lower et al(1996)) and lower grade tumours (which are in turn associated with ER positivity(Fisher et al(1981); Fisher et al(1980))) have been reported (Harding et al(1995)). However, one might also expect that tumours presenting in younger, pre-menopausal women were more commonly hormone-sensitive, but in fact the opposite is the case. Thus the mechanism for the relationship between oestrogens and the increased risk of breast cancer remains unclear.

Hormonal Treatment of breast cancer

With the hormonal milieu clearly being of importance in the risk, and possible promotion, of breast cancer, it is natural that attempts should be made to alter that milieu to try and control the growth of an established breast cancer. The partially successful attempt by Beatson of controlling the disease with oophorectomy (Beatson, (1896)) in fact constitutes the first report of systemic therapy for any cancer. A subsequent report confirmed responses for this form of hormone therapy in about one third of a group of pre-menopausal women (Boyd, (1900)), a figure consistent with current experience of hormone therapy in similar patients with unknown tumour ER concentration.

During the 1950's, there was clinical interest in the use of oral anti-oestrogens to control advanced breast cancer, in order to reduce the side-effects associated with the alternatives of surgical (or radiotherapeutic) oophorectomy and surgical adrenalectomy. Clomiphene was found to be effective, but too toxic for prolonged use (Herbst et al(1964)). I.C.I. had developed the anti-oestrogen 1-(p-dimethylaminoethoxyphenyl)-1,2-*trans*-diethyl-but-1-ene, (ICI 46,474 or Tamoxifen) for use as a possible oral contraceptive (Wiseman, (1994)). It was therefore also tested in metastatic breast cancer, and found to be effective and well-tolerated (Cole et al(1971)). In parallel, several studies were also undertaken which confirmed its efficacy in the DMBA-induced rat mammary carcinoma, the only hormone-dependent animal tumour model available at that time (Jordan, (1974); Nicholson et al(1975)).

Pre-operative therapy for non-metastatic breast cancer

Following the demonstration that a significant proportion of cases of advanced breast cancer responded to tamoxifen, further studies were conducted to test the possibility that post-surgical adjuvant administration of tamoxifen might improve survival, which has indeed been confirmed by the most recent meta-analysis (Early Breast Cancer Trialists Collaborative Group, (1992)). Whilst the drug continues to be a mainstay of endocrine therapy for breast cancer, including advanced disease (Jaiyesimi et al(1995)), in all situations in which it is employed, treatment failures occur. When used in the adjuvant setting for early, potentially curative, disease this is only apparent at relapse, at which point cure is no longer a realistic possibility. However there has been increasing use of primary systemic therapy for non-metastatic breast cancer, where chemotherapy and/or endocrine therapy is administered first, before definitive loco-regional treatment. Most studies report the use of chemotherapy, and therefore this will be considered first to illustrate the potential of this indication for drug therapy.

Pre-operative chemotherapy

Pre-operative or “neo-adjuvant” systemic therapy has been used for locally advanced breast cancer for at least two decades. The rationale for its use was the concept that effective cyto-reduction would render the primary cancer more amenable to surgery, and improve the otherwise rather poor long-term survival of women with this type of disease. The impact of systemic treatment on local control is significant, but it rarely translates into improved survival. An EORTC study found that the combination of CMF and hormones (ovarian irradiation and prednisolone for premenopausal women or tamoxifen for post-menopausal) administered after radiotherapy gave a significantly better local control rate but no significant survival advantage (Rubens et al(1989)). A similar benefit in local control for the use of combined hormone and chemotherapy was noted in a retrospective analysis (Perez et al(1994)).

There have been many studies involving different schedules and drugs, but it has been much easier to improve response rates than survival; and as has been found in metastatic disease, as well as laboratory models (DeVita, Jr. (1993)), a higher response rate does not always translate into improved survival. However there are clinical data to suggest that the pathological response may be clinically important ; those patients whose tumours that have gone into a pathological complete remission have a better survival (Bonadonna et al(1993); Feldman et al(1986)), and for all patients the principal determinant of a worse long-term prognosis is the number of ipsi-lateral axillary nodes that are found to contain metastases at the time of surgery (Attia-Sobol et al(1993); Gardin et al(1995); Gröhn et al(1984)).

With this experience of primary systemic therapy in locally advanced disease, and the data from laboratory models demonstrating an apparent proliferative stimulus to other sites of disease upon removal of the primary tumour (Holmgren et al(1995)), (which can be prevented by prior therapy with cyclophosphamide or ovarian ablation (Fisher et al(1989))), there has been increasing interest in the use of chemotherapy before surgery in patients with operable breast cancer (Epstein, (1996)). This has the twin advantages of increasing the potential for breast conservation (Powles et al(1995)), as well the theoretical possibility of an improvement in survival. However this latter aim has yet to be realised, and although two randomised trials have reported a benefit (Mauriac et al(1991); Scholl et al(1994)), in one case this was lost with further follow-up (Scholl et al(1995)), and in other 10% of those relapsing in the control arm had had no adjuvant therapy (Mauriac et al(1991)). Thus any survival advantage for pre-operative therapy over conventional post-operative adjuvant treatment is likely to be small. Furthermore several studies have confirmed that despite a response in the primary tumour conferring a survival advantage (Bonadonna et al(1993); Jacquillat et al(1990); Scholl et al(1995); Williams et al(1988)), the more profound influence on the subsequent clinical course is

again the number of ipsi-lateral involved axillary nodes (Cameron et al(1997); Frye et al(1995); Jacquillat et al(1990)).

Pre-operative hormone therapy

In contrast there are few published data on the use of hormone therapy for locally advanced disease, although a study from Nottingham (Robertson et al(1994)) which compared primary tamoxifen and radiotherapy (with cross-over on progression) found no difference in survival between these two modalities. Similarly a randomised study conducted by the E.C.O.G. found that there was not only no detriment, but possibly an advantage, in treating elderly patients with tamoxifen first, and withholding chemotherapy (in the form of CMF) for tumours progressing on tamoxifen (Taylor, IV et al(1986)). However neither of these studies restricted the use of tamoxifen to ER positive tumours, and as such they are not a fair representation of the current practice in many centres. In early, or operable breast cancer, there are similarly few reports of primary endocrine therapy, with most of the available data coming from elderly women where therapeutic minimalism is usually the goal (Allan et al(1985)) - which may no longer be justified (Dixon, (1992)). It is clear however that excellent results can be obtained - a 5-year survival rate of over 90% was reported in one study for those women who had had a clinical complete response to tamoxifen (Horobin et al(1991)). However a poorer response was associated with a worse survival, although in another study, a measure of ER was a better predictor of medium-term survival than was the clinical response after either 3 or 6 months' therapy (Gaskell et al(1992a)). With women under the age of 70, there is less experience of hormone therapy, particularly in patients with operable breast cancer. The largest series is from the Edinburgh Breast Unit (Anderson et al(1991)), where pre-operative hormone therapy was initially offered to all women with large primary breast cancer. However after no responses were observed in tumours with an ER concentration of less than 20 fmol/mg cytosolic protein, pre-operative hormone therapy was confined to patients with ER positive tumours (Anderson et al(1989)). The long-term follow-up of these women has been recently published, and the 10 year actuarial survival is no different for those women whose tumours responded to pre-operative chemotherapy or hormone therapy (Cameron et al(1997)). This was not however a randomised study, so no firm conclusions can be drawn, but it suggests that effective hormone therapy would appear not to be detrimental in comparison with doxorubicin-based chemotherapy.

Response rates to tamoxifen vary between published series, depending on the stage of disease and types of tumours treated. It is universally accepted however that the presence of Oestrogen Receptors is associated with a 60 - 70% response rate to tamoxifen (Allan et al(1985); Allegra et al(1980); Brooks et al(1980); Osborne et al(1980); Williams et al(1987)). However cut-off values for positivity can vary between 5 & 20 fmol/mg cytosolic protein ; the latter having clinical meaning as no

significant responses were seen in tumours with values below that level after three months pre-operative hormone therapy (Anderson et al(1989)). Although a lack of Oestrogen receptors predicts for tamoxifen insensitivity (Anderson et al(1989); Robertson et al(1994)), there are data to suggest that up to 10% of ER negative/poor tumours may sometimes respond to tamoxifen (Allegra et al(1980); Williams et al(1987)) or indeed other hormone therapies (Dao et al(1980)). Further refinement of the prediction of response to hormone therapy has been attempted by the additional measurement of progesterone receptor (PgR) concentration. Undoubtedly the response rate is higher for tumours that express both ER and PgR, with rates of 66 - 83% reported (Brooks et al(1980); Dao et al(1980); Osborne et al(1980)). However a policy of only giving hormone therapy to ER and PgR positive tumours would still fail to induce a response in 20% of patients' tumours, and would also exclude the 30 - 60% of ER positive/PgR negative tumours who would have responded to such treatment (Brooks et al(1980); Dao et al(1980); Osborne et al(1980)). PS2, another oestrogen-inducible protein has also been considered for its utility as an indicator of oestrogen-sensitivity (Soubeyran et al(1996a)), but as with PgR, there are a significant minority of tumours expressing both ER and pS2 that do not respond.

There are data to suggest that the concentration of ER can help predict for endocrine-sensitivity, with response rates being significantly better in tumours with higher levels of ER (Gaskell et al(1992a); Osborne et al(1980)) ; however limiting the use of hormone therapy to those patients whose tumours have a high level of ER (such as > 100 fmol/mg) would not only exclude a significant proportion of patients with responsive tumours, but could exclude the patients with the best long-term prognosis as there are data suggesting that the prognosis for women whose tumours have a particularly high ER value may be worse than those with a lower, but still positive value (Sancho-Garnier et al(1995); Thorpe et al(1993)). Finally a high proliferative fraction may predict for a poorer response despite ER-positivity (Nicholson et al(1991)), and there is one study suggesting that bcl-2 expression might be a more specific marker than ER of a hormone-sensitive tumour (Gee et al(1994)).

In conclusion therefore, pre-operative therapy with tamoxifen is in routine clinical use, although largely for elderly women. There are however data to suggest that its use could be extended to other patients, but accurate predictors of response would greatly benefit this approach. Long-term survival is associated with a complete pathological response to therapy, be it in the primary tumour or the axillary nodes. Residual disease in the breast possibly reflects resistant disease, whereas persistent axillary nodal involvement may not only reflect resistance but also an increased risk of systemic disease which is itself potentially resistant. However current knowledge does not seem to be able to predict tamoxifen response with a sensitivity of more than 80%.

Failure of tamoxifen therapy

Clinically tamoxifen is administered for considerable lengths of time, and laboratory studies confirm its long-term efficacy (Osborne et al(1987)). However the undoubted benefit of adjuvant tamoxifen continues to be marred by the continual relapse of patients whilst still taking the drug. Thus one can consider that there are likely to be two populations of patients, as in the metastatic setting : those whose tumours progress despite tamoxifen, and those in whom an initial response gives way to secondary resistance. Further laboratory and clinical work has been conducted toward determining the precise mechanism of action of the drug as well as identifying phenotypes that are primarily resistant. Confirmation that the existence of both primary and secondary tamoxifen resistance is not confined to patients with overt metastatic disease comes from studies of tamoxifen administered to patients with non-metastatic breast cancer. Furthermore unsuccessful tamoxifen therapy not only exposes a woman to the toxicity of the drug, which includes the rare but serious problem of endometrial carcinoma (Barakat, (1995)), but might be prejudicial to her overall therapy. Some patients may receive tamoxifen in addition to cytotoxics, so it could be argued that inefficacy is less significant than when used as monotherapy. However there certainly are at least *in vitro* data demonstrating antagonistic interactions between tamoxifen and commonly used cytotoxics, including 5-fluorouracil, and possibly cytophosphamide (Osborne, (1994)). In contrast there is a suggestion that tamoxifen might have a synergistic action with doxorubicin (Osborne et al(1989)), although an antagonistic interaction has also been reported (Hug et al(1985)). There are other reasons to be concerned about the concomitant administration of cytotoxics and tamoxifen, which has not been demonstrated to be advantageous as compared with sequential administration. Firstly, as will be discussed subsequently (see page 36), tamoxifen appears to reduce the proliferative index of tumours, irrespective of their ER status (Clarke et al(1993)), and (untreated) tumours with higher proliferative fractions are more sensitive to chemotherapy (Remvikos et al(1993)). Furthermore there are data, generated from the clinical tumours studied in this thesis, that tamoxifen administration can result in increased p-glycoprotein expression, particularly in non-responders (Keen et al(1994)). However the clinical results are mixed : a large retrospective review of patients with metastatic disease found that prior hormone insensitivity prejudiced survival when cytotoxics were subsequently administered (Swenerton et al(1979)), whereas another reported no detrimental effect (Gregory et al(1993)). A randomised cross-over study in locally advanced disease and metastatic disease, which did not select therapy on the basis of ER status, found a trend towards a better outcome if patients were given tamoxifen first, as compared with those given initial CMF chemotherapy (Taylor, IV et al(1986)). Finally the Edinburgh Breast Unit's experience in early breast cancer is that the response rate to doxorubicin-based chemotherapy was possibly worse in those patients given prior hormone therapy.

with only 14/22 (64%) responding as opposed to the 24/28 (86%) given primary chemotherapy (Cameron et al(1997)), and their survival appeared worse ($p = 0.1$, unpublished data).

In summary therefore, earlier and more accurate prediction of tamoxifen sensitivity or resistance could be of immediate clinical benefit.

Aims of this study

Tamoxifen has an important rôle in the treatment of breast cancer. However it is not universally efficacious, and a failure to respond to tamoxifen might even be counter-productive in some tumours.

The aims of this study were therefore to :

- i. examine biological changes in clinical breast cancer occurring during tamoxifen therapy.
- ii. correlate these changes with tumour response.
- iii. confirm the timing of these changes in a model system.
- iv. confirm the specificity of these changes to tumour response in the model system.

In order to facilitate the translation of these findings to clinical practice, the methodologies chosen had to be reproducible when applied to clinical specimens. Hence techniques such as flow cytometry and immunohistochemistry were chosen in preference to in-situ RNA detection or electrophoretic gels.

Potential markers for tamoxifen response

Predicting exactly which tumours will respond to tamoxifen is, has already been stated, difficult. A lack of expression of oestrogen receptors identifies tumours with only a low probability of response, but it is less clear as to what is the best method of predicting which ER positive tumours will respond to hormone therapy. The markers considered, including pS2 and PgR, have already been discussed, and there is a need to identify other indicators of potential resistance to tamoxifen for ER positive tumours. Such markers need not just be static, but could be dynamic, where the evidence of resistance to therapy is reflected in the changes in tumour biology that occur with therapy. Potential biological characteristics of breast cancers will therefore be considered, and the current knowledge of their interaction with tamoxifen discussed.

Oestrogen Receptors

Given that ER positivity is consistently the best predictor of hormone sensitivity in breast cancer, it is possible that any changes in expression that occur with therapy might improve upon the utility of this protein for identification of tamoxifen resistance.

Expression of the Oestrogen receptor appears to be under a negative feedback control, with inhibition of the mRNA for the ER occurring upon exposure to oestrogen (Wiseman, (1994)). Anti-oestrogens such as 4-hydroxytamoxifen could block this effect. One might therefore expect that effective anti-oestrogen therapy would be associated with increased expression. However if hormone therapy is effective, then it could selectively reduce the proportion of breast cancer cells that are ER positive, leaving a greater proportion of the tumour as ER negative, and thus a repeat measurement of ER might result in a lower level of expression. Ineffective therapy would therefore be expected to result in no change, but the direction of change in responding tumours remains unclear. The data from clinical studies are mixed - with some reports of a decreased level of expression after endocrine therapy (Allegra et al(1980); Lumsden et al(1997); Taylor et al(1982)), and others finding no overall change (Hawkins et al(1990); Hull et al(1983)). The negative study reported by Hawkins et al (Hawkins et al(1990)), did note that the ER concentration fell after treatment in three patients treated with tamoxifen, but not with other forms of pre-operative endocrine therapy, and suggested that some of the discrepancy in the clinical studies could be due to the known interference of bound tamoxifen with the DCC assay (Hull et al(1983)), and concluded that a change in ER was not the mechanism of action of endocrine therapy for breast cancer. Consistent with this was the small rise in ER concentration seen in the benign uterus during therapy with the LHRH agonist zoladex (Lumsden et

al(1997)), a trend also observed when this agent is used in the pre-operative treatment of breast cancer (Hawkins et al(1990)).

Proliferation

Given the relationship between oestrogens and both the incidence of breast cancer and the biology of the normal breast epithelium, it is important to develop further the link between these hormones and the behaviour of both the normal and malignant breast. Oestrogen exerts its effects at the cellular level by binding towards the C-terminal end of a nuclear protein, the oestrogen receptor, which then dimerises (Kumar et al(1988)) and interacts with a specific DNA sequence, the ERE (Green et al(1988)). Subsequent upon this binding, transcriptional activation of oestrogen-induced genes is mediated by two regions ; AF-1, which appears to be constitutively functional, and is located in the N-terminal region, and AF-2 which is in the hormone-binding domain and depends on ligand binding for its functionality (Parker, (1994)). Oestrogen-stimulated proliferation, which usually requires other mitogens like insulin or Insulin-like Growth Factor-1 (IGF-1), acts early in G₁; in contrast progestogens stimulate and both anti-oestrogens and anti-progesterones inhibit cell proliferation later in G₁ (Sutherland et al(1992)). Shortly after exposure to a growth stimulant, there is evidence of increased expression in breast cancer cells of various genes, including c-fos and then c-myc (Sutherland et al(1994)). Induction of Cyclin D1 also occurs in early G₁, but this is transient, and levels fall as the cell progresses through the cell cycle (Sutherland et al(1994)). Cyclin D1 appears to be rate-limiting for the transition from G₁ to S phase (Musgrove et al(1994)), and can be inhibited by cyclin-dependent kinase inhibitors such as p21^{WAF-1/Cip-1} (Harper et al(1993); Xiong et al(1993)). Further evidence of the potential importance of cyclin D1 in breast cancer comes from a recent study which reported that the incidence of over-expression rose from 18% of atypical ductal hyperplasia (ADH) (a benign proliferative lesion with only a five-fold increased risk for breast cancer) to over 75% in ductal carcinoma-in-situ or frank invasive carcinoma (Weinstat-Saslow et al(1995)). Studies using immunohistochemistry have found however that only one-third of breast cancers over-express the gene product (Bartkova et al(1994); Gillett et al(1994)). The observation that those clinical breast cancers that overexpress cyclin D1 are more likely to be ER-positive (Buckley et al(1993); Fantl et al(1990)) is consistent with its involvement in oestrogen-stimulated growth. Furthermore over-expression or gene amplification of cyclin D₁ may predict for a worse outcome (Schuuring et al(1992)), particularly when associated with high levels of the retinoblastoma protein (pRB) or the epidermal growth factor (EGFR) (McIntosh et al(1995)) ; this may be because increased cyclin D1 expression appears to enhance amplification of other genes, at least in an experimental situation (Zhou et al(1996)).

There are many potential surrogate markers of increased proliferation within a breast cancer. During the S-phase of the cell cycle, DNA is synthesised. Thus measuring the uptake of DNA-precursors permits an assessment of the proliferative fraction of breast cancer cells. The standard method has been to measure the proportion of cells that became radioactive following the administration of ^3H -thymidine, expressing the result as the Labeling index (LI) (Meyer et al(1988)). However this method requires the use of radioisotopes, is not practical in patients or fixed tissue, and has thus been largely superseded by other techniques. A non-radio-isotope variation of this method using bromo-deoxy-uridine (BrdU) is available (Lloveras et al(1990)), but is also limited by the requirement for the cells to be viable. Techniques that rely on active DNA synthesis are impossible in fixed cells, but alternative methods are becoming available. Estimation of the S-phase fraction by flow cytometry (for details see the methods section) permits measurement of the proportions of cells in the various phases of the cell cycle, as identified by their DNA content; thus cells in G_0 or G_1 are distinguishable from those in S-phase and those in G_2 or M. However discrimination between resting G_0 and actively proliferating G_1 cells is not possible by this methodology alone, nor can the technique separate pre-mitotic G_2 cells from those in mitosis. Furthermore the actual proportion of cells actively proliferating cannot be estimated because of the inability to identify G_1 cells, and has to be estimated by multiplying the S-phase fraction by 3 (Meyer et al(1986); Sahin et al(1991); Wintzer et al(1991)). Whilst good results can be obtained from disaggregated cells grown in flasks or obtained from tumours by Fine Needle Aspiration, when used on paraffin-embedded material the method is less reliable (Hedley, (1989)).

For solid tumours which are often fixed and processed into paraffin-wax blocks it has been necessary to assess other markers of proliferation. The classic measure is the mitotic index. Cells that are in mitosis are easily identified as having absent nuclear membrane (i.e. the cells have passed prophase), "clear hairy extensions of nuclear material" (i.e. chromosomal condensation), which are either clotted (early metaphase), in a plane (metaphase/anaphase) or separated (telophase) (Jannink et al(1996)). Indeed the total number of mitotic figures in 10-high power fields is one of the three cardinal features used to assess the grade of a clinical breast cancer (Bloom et al(1957); Elston, (1984)), and the mitotic index has been shown to be assessable with reproducibility (Clayton, (1991); van Diest et al(1992)). Alternatively, by using antibodies, immunohistochemistry allows the identification of cells that express antigens associated with proliferation. A number of proliferation-associated antigens are known - including Ki-67, which is expressed throughout G_1 , S & G_2 /M (Gerdes et al(1984)), PCNA (Mathews et al(1984)) which accumulates in S-phase (Ogata et al(1987)) and Ki-S1 (Kreipe et al(1993)) which is topo-isomerase I (Boege et al(1995)). That these are all measures of proliferation is confirmed by the correlations between PCNA and thymidine-labelling (Battersby et al(1990)), DNA-flow cytometry (Garcia et al(1989)) and Ki-67 (Dawson et

al(1990); Gasparini et al(1994a)) : between S-phase fraction and Ki-S1 (Camplejohn et al(1993)), Ki-67 (Dawson et al(1990); Gasparini et al(1994a); Keshgegian et al(1995)) and inconsistently PCNA (Dawson et al(1990); Gasparini et al(1994a); Keshgegian et al(1995)) : but poor correlations were found between S-phase fraction and BrdU labelling (Lloveras et al(1994)). However, there are data to suggest that both PCNA and Ki-S1 staining may vary with different concentrations of antibody, whereas the MIB-1 antibody for Ki-67 (which is associated with several other proliferative markers (Keshgegian et al(1995)) including the mitotic index (Weidner et al(1994))) is more robust and is not expressed in non-proliferating cells (McCormick et al(1993)).

c-erb-B2

In the same manner that oestrogen can drive benign and malignant breast cell proliferation via the oestrogen receptor, there are other families of growth factors and their receptors that appear to play important rôles in the biology of breast cancer. One of the most frequently implicated receptors are members of the class I receptor tyrosine kinase family, which includes c-erb-B2 or HER-2/neu (Coussens et al(1985)). In vitro studies suggest that whilst the expression of c-erb-B2 is inhibited by oestrogen (Dati et al(1990)), is upregulated by tamoxifen (Antoniotti et al(1992)) in cell lines (including ZR-75-1) that are sensitive to tamoxifen's anti-proliferative effect. In contrast tamoxifen had no effect on growth or c-erb-B2 expression in an insensitive cell line, MDA-MB-231. The true relationship between growth regulation and c-erb-B2 expression is however more complex, as not all compounds that are growth-inhibitory are associated with altered c-erb-B2 expression. Using the T47-D and ZR-75-1 ER-positive cell lines, it has been shown that in addition to the previously observed effects of oestrogen and tamoxifen on expression c-erb-B2, progesterone increased expression whilst inhibiting growth, whereas prolactin had a similar anti-proliferative effect without any observed change in expression at either the message or protein levels (Taverna et al(1994)). A direct link between the two receptor pathways, ER and c-erb-B2 was recently confirmed, which goes some way to clarifying their relationship in clinical breast cancer. In a study employing MCF-7 ER-positive breast cancer cells, some of which had been transfected with the gene encoding for c-erb-B2, it was clearly demonstrated that the c-erb-B2-transfected cells were no longer dependent on oestrogen for growth, and were insensitive to tamoxifen's growth inhibitory effects, when grown both in cell culture and nude mice (Pietras et al(1995)). This change in tamoxifen-sensitivity came about without any alteration in the intra-cellular tamoxifen concentration, and could be reversed by the addition of a monoclonal antibody to the c-erb-B2 protein, rhuMAb HER-2. The interaction between the two receptor pathways was confirmed by the increased phosphorylation and ERE binding of the ER upon growth stimulation in the transfected cells by heregulin, a peptide growth factor that binds to the c-erb-B2 receptor (Carraway et al(1994)). That this implicates a true post-receptor oestrogen-dependent pathway was confirmed by the increased PgR expression observed, confirming that the c-erb-B2 receptor can induce changes in genes that are under the normal control of oestrogen. Furthermore prolonged exposure to heregulin resulted in a significant decrease in the level of both ER and PgR expressed, which could thus itself give rise to a hormone-insensitive cell.

In clinical breast cancer, c-erb-B2 was first recognised to be of importance as a prognostic factor. Gene amplification resulting in over-expression occurs in up to a third of breast cancers (Barnes, (1989); Slamon et al(1987)), and is associated with a poor prognosis in lymph-node positive tumours (Borg et al(1991); Schönborn et al(1994); Slamon et al(1987); Têtu et al(1994)) although the

association is less-well defined in lymph-node negative tumours (Borg et al(1991); Gasparini et al(1993); Gullick et al(1991)). Several studies suggest that over-expression correlates with other poor prognostic features, such as high proliferation as measured by S-phase fraction (Borg et al(1991)) or Ki-67 (Sirvent et al(1994)), tumour grade (Schönborn et al(1994); Sirvent et al(1994)) and the absence of steroid receptors (Borg et al(1991); Sirvent et al(1994)) ; although other studies do not always find such associations (Lönn et al(1994)). Some of these discrepancies may be due to methodological discrepancies such as different fixatives (Penault-LLorca et al(1994)), or antibodies (Press et al(1994)). The data relating c-erb-B2 overexpression or gene amplification (which appear to not be synonymous (Barnes, (1993))) with the absence of steroid receptors highlights an important relation between these two classes of receptors, which is further confirmed clinically by report of upregulation in tamoxifen-treated clinical cancers (Johnston et al(1993)), consistent with in vitro data. This effect, which on first thought appears counter-intuitive to the anti-proliferative effect of tamoxifen, could therefore be an explanation for the observation, that at least in some series, c-erb-B2 positive breast cancers are hormone-insensitive, even when expressing ER (Allred et al(1992); Gusterton et al(1992)), although not all authors confirm these findings (Archer et al(1995); Barnes, (1993)). Indeed, there is a study suggesting that c-erb-B2 positive tumours might actually do worse when given tamoxifen, as compared to controls, the majority of whom were in control arms of adjuvant trials and thus received no post-surgical systemic therapy (Borg et al(1994)). Furthermore an association has been reported in ER-positive breast cancer between c-erb-B2 expression and proliferation (Borg et al(1991)).

Thus although studies suggest that the relationship between c-erb-B2 and oestrogen is such that the c-erbB2- pathway is, upon stimulation, capable of bypassing the dependency of the ER upon its natural ligand oestrogen, the data on the effect of tamoxifen on c-erb-B2 expression suggest that it could be a candidate marker for early detectable changes with tamoxifen treatment. As with most oncogenes, there are several ways in which levels of expression can be detected, but as stated above the intention was to define a marker of tamoxifen-response that was available both in tissue and flow-cytometry. Therefore, as for proliferation, the most suitable is immunohistochemical detection with a monoclonal antibody, because the same methodology can be transferred to FNAs analysed by flow cytometry simply by employing fluorescein-tagged secondary antibodies (Stål et al(1994)). Furthermore, although detection of amplified mRNA levels has been reported using the flow cytometry (Li et al(1994)), the discrepancies sometimes reported for c-erb-B2, both in vitro (Taverna et al(1994)) and in vivo (Barnes, (1993)), between levels of mRNA and protein suggested that changes in protein expression would be more relevant as it is the receptor itself that is involved in the cellular changes associated with c-erb-B2.

Apoptosis & cell-death

Growth depends on the relative balance between cell production and cell loss (Schwartzman et al(1993)). Cell death was traditionally considered to be a pathological process, caused by a hostile environment, resulting in necrosis (reviewed in (Wyllie, (1981))). It can occur in response to several insults, including ischaemia (Borgers et al(1987)), and metabolic poisons (Trump et al(1984)), and is associated with distinctive changes : initial swelling of the cytoplasm with only minor changes in the nucleus. Subsequently there is organelle and plasma membrane disruption, and the intracellular contents spill into the extra-cellular space (Trump et al(1982)). Proteolytic digestion of histones results in exposure of the DNA which is then cleaved into fragments of varying sizes (Duvall et al(1986)). This process is typically seen in contiguous cells, and may be associated with hemorrhage and or an inflammatory response.

However there is another, more controlled, form by which a cell can die. This programmed or physiological cell death has been increasingly realised to play a major rôle in tissue homeostasis, and is prevalent in many tumours. It occurs rapidly, and in contrast to necrosis, usually affects scattered cells (Bellamy et al(1995)). Its morphology was first described in 1907 (Collin, (1906)) but it was not until 1972 that various reports of these observations were recognised to be examples of the same process, which was given the name of apoptosis (Kerr et al(1972)), from the Greek word for leaves falling off trees. Morphologically, apoptotic cells are quite distinct from necrotic ones, and this is discussed in the review referred to above (Wyllie, (1981)). Apoptosis usually affects single or small numbers of cells in an asynchronous fashion. Under the light microscope the first visible feature of this process is cytoplasmic condensation, which is apparently associated with loss of intracellular fluid (Lockshin et al(1981)). There is a loss of intercellular contact, and the cell is also released from the basement membrane. Intermediate filaments condense around the nucleus (Wyllie, (1987)), and the cell is isolated from its neighbours. This process can be very rapid, occurring in a few minutes (Tenniswood et al(1992)). The next stage, which remains visible for perhaps a few hours, consists of chromatin condensation which results in nuclear blebbing (Cohen, (1993)), which occurs concomitantly with activation of an endogenous Ca^{2+} dependent endonuclease which cleaves the DNA, often into lengths that give rise to the typical appearance of the "DNA ladder" on agarose gel electrophoresis (Arends et al(1990)). Morphologically the typical apoptotic cell has several membrane-bound dense pyknotic nuclear fragments known as apoptotic bodies, which are then usually phagocytosed either by macrophages or adjacent cells (Wyllie, (1993)). Perhaps because DNA fragmentation into the classic "ladder" is so easily detected, it has been proposed as the "hallmark" of apoptosis (Compton, (1992)). However DNA ladders cannot alone distinguish between apoptosis and necrosis, since they have been seen following hepatic necrosis induced by

paracetamol (Ray et al(1993)), and necrosis of murine mastocytoma cells caused by DEAE-Dextran (Collins et al(1992)). Furthermore there are reports of apoptosis, detected morphologically, that are not associated with DNA laddering (Cohen et al(1992); Thompson, (1991)). It is therefore the morphological appearance of an apoptotic cell that remains its "hallmark" (Wyllie, A.H., personal communication).

Much of the understanding of apoptosis has come from work in lymphoid cell systems, which may not be immediately relevant to epithelial tissues such as the breast. Some of the triggers that induce apoptosis are beginning to be understood, and include APO-1, or *fas*, (Itoh et al(1991)), TNF and other members of the same family (Fehsel et al(1991); Kyprianou et al(1991a)). There is also evidence for a third pathway involving ceramide and perhaps Protein Kinase C as an inhibitor (discussed in (Schwartzman et al(1993))). Downstream the final common pathway appears to involve cysteine proteases of the Interleukin-1 β converting enzyme (I.C.E.) family, whose actions include cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (Nicholson et al(1995)). Various regulatory genes have been identified, including *c-myc*, *bcl-2* and other members of the same family, and p53 ; some will be discussed in more detail. The importance of *c-myc* is that it is associated with increased proliferative stimulus to the cell, but if there are insufficient growth factors, also increased apoptosis (Evan et al(1992)). In contrast, Ha-ras transfection induced a similar proliferative drive but without the increased propensity to undergo apoptosis (Arends et al(1993)). Other interventions that cause cell-cycle block, such as topo-isomerase II inhibition or thymidine-induced S-phase arrest, also induce apoptosis and thus appear to mimic the effect of serum starvation (Bertrand et al(1991); Fanidi et al(1992)). These and other data suggest that at least one group of cells that are primed for apoptosis are those in a high turnover state (Wyllie, (1993)), an observation that will have relevance when examining the evidence for apoptosis in breast epithelial cells. There is no doubt that p53 plays a major rôle in the induction of apoptosis in many cell systems ; this is discussed elsewhere (vide infra). However apoptosis can occur without p53 being involved, and in a classic pair of studies it was shown that in lymphoid cells p53 is necessary for etoposide (a potent topo-isomerase II inhibitor) and γ -ray induced apoptosis, but not for that consequent upon glucocorticoid exposure (Clarke et al(1993)). Furthermore there is evidence that androgen ablation can induce p53-independent apoptosis in prostatic glandular cells (Berges et al(1993)), and in human prostate cancer xenografts (Kyprianou et al(1990)) in which it appears to be associated with increased TGF β_1 expression.

Apoptosis occurs in the normal breast epithelium (Ferguson et al(1981)), and the frequency with which it is seen varies throughout the menstrual cycle, with a peak occurring only a few days after that of proliferation (Anderson et al(1982)). Having proliferated during pregnancy, mammary tissue

in the rodent then involutes at the time of weaning, with induction of apoptosis in the glandular epithelial cells resulting in the loss of 75- 85% of the cells (Battersby et al(1988); Walker et al(1989)). Control of these processes is through a complex set of hormonal interactions, although it is not possible to determine which specific hormone is responsible. It is perhaps therefore not unexpected that ovarian ablation can cause apoptosis in breast tumours (Warri et al(1993)).

The gold standard measure of apoptosis is the characteristic apoptotic cell - with its contracted eosinophilic cytoplasm, condensed chromatin with or without nuclear blebbing, occurring in a single cell asynchronously with other cells in the vicinity that may be undergoing the same process (Bellamy et al(1995)). However identification of such cells in a tissue section is time-consuming, and potentially subject to inter-observer variation. Furthermore the low incidence of apoptosis in breast cancers (Lipponen et al(1994)) heightens the possibility that there could be significant intra-tumour variation, depending on the section examined, unless a large number of cells were to be examined. Because of these limitations other techniques have been investigated in the hope that they might be more sensitive and less subject to variation. For cell line experiments, many workers have separated extracted DNA on an agarose gel, citing the characteristic DNA ladder as proof of apoptosis. When the total number of cells are known, quantification of the ladder DNA permits comparison of the apoptotic frequency between experiments. However for clinical breast tumours, only a proportion of the extracted DNA will have come from the invasive cancer cells, with unknown proportions also being contributed by stroma, benign ducts, D.C.I.S. and infiltrating leucocytes. Indeed in a study of hormone depletion in rodent tumours, in which the presence of apoptotic cells had been confirmed, the proportion of the DNA that was fragmented was too small to be visualised by this technique (Wijsman et al(1996)). Thus without a detailed analysis of the relative cellularity of the specimens, comparisons are almost impossible.

However the occurrence of DNA fragmentation can be used immunohistochemically to identify cells that contain DNA strand breaks. There are essentially two different techniques in use, which only vary in the enzyme used to identify free DNA ends. In Situ Nick Translation was first used in cultured cells (Fehsel et al(1991); Kishimoto et al(1990)), and subsequently adapted for cell suspensions and tissue sections (Gold et al(1993)). The principle of the method is that DNA polymerase will extend free 3' ends of DNA. If the cells are incubated with DNA polymerase and dUTP labeled with either fluorescein or digoxigenin (which is then further incubated with fluorescein-labeled anti-digoxigenin antibodies), any free DNA ends will be labelled with these marked nucleotides, which will be visible under a fluorescent microscope. The alternative method uses the enzyme terminal deoxy-transferase (Gavrieli et al(1992)), and similarly labeled dUTP nucleotides. Both these techniques have the advantage that they can be used on either cell

suspensions or tissue sections, and that both positive and negative controls can be performed (by respectively pre-incubating with DNAase or omitting the basic enzyme). However they are essentially indirect techniques for measuring DNA damage, and as discussed above, and in a recent review (Darzynkiewicz et al(1992)), cannot be relied upon as the only form of identification of an apoptotic cell. Necrotic cells may also stain with these techniques, although the actual intensity of the stain may help distinguish between necrotic and apoptotic nuclei. Studies which have compared TUNEL and ISEL have shown that whereas the former was more sensitive for the early stages of apoptosis, earlier identification of necrotic cells occurred with the latter (Gold et al(1994)). Furthermore, in a small study in clinical breast cancer, the two techniques were noted not to be synonymous (Azam et al(1994)), with TUNEL being the more sensitive technique for detecting morphologically obvious apoptotic cells. Advocates of the use of these techniques in the identification of apoptotic cells also cite another beneficial argument, namely that they can detect cells at an earlier stage of apoptosis (Gavrieli et al(1992); Umansky, (1982)) and thus potentially increase the sensitivity of detection. However in one study of breast cancers, there were also cells that were clearly apoptotic by morphological criteria that were not identified by the TUNEL method (Dowsett et al(1995)). Problems using the TUNEL assay to distinguish between apoptosis and necrosis have also been reported in other systems (Grasl-Kraupp et al(1995); Wijsman et al(1996)).

For cell suspensions there are other methods of identifying apoptotic cells. Firstly, apoptotic cells often have reduced DNA staining with various fluorochromes, giving rise to the characteristic sub-G1 peak using a flow cytometer (Darzynkiewicz et al(1992)). It remains unclear whether this is due to reduced DNA accessibility to the fluorochrome, or reduced DNA content - although the latter explanation is more likely, since different fluorochromes give rise to the same sub-G1 peak, and studies on DNA in fixed and unfixed apoptotic cells suggest that a considerable proportion of the DNA in apoptotic cells is of low molecular weight, and dissociates from cells whose membranes have become more permeable secondary to the fixation process (Arends et al(1990)). The disadvantage of relying on a sub-G1 peak to identify apoptotic cells in needle aspirates from xenografts grown in nude mice, is that any cells of a mouse origin (such as leucocytes, vascular endothelial cells and stroma) will have a reduced DNA content as compared with the human-derived tumour cells, and will thus themselves also form a sub-G1 peak ! (See figure 1). Furthermore any other DNA fragments from necrotic cells could blur the pre-G1 region of the DNA histogram, rendering measurement of changes in sub-G1 peaks difficult, unless they are of considerable size.

A potentially more useful technique relies on the fact that unfixed apoptotic cells retain integrity of their plasma membranes, unlike necrotic cells (Lennon et al(1991)). However such dye-exclusion assays will only be able to also differentiate between live and apoptotic cells by using a second dye

that assesses viability such as Hoechst 33342 (Pollack et al(1991)), which requires excitation by a laser at a different frequency from many that are widely available. A further refinement of the method is to pre-incubate with trypsin and DNAase I in order to completely disrupt the necrotic and late apoptotic cells which cannot exclude these enzymes (Darzynkiewicz et al(1984); Potmesil et al(1983)). Finally there is evidence that apoptotic cells have a different flow cytometric appearance as assessed by their forward (a measure of size) and side (a measure of granularity) scatter characteristics, when examined in conjunction with their DNA-binding to propidium iodide and Hoechst 33342 (Gold et al(1993)). Apoptotic cells are smaller, with lower forward scatter, and marginally more granular with higher side scatter. However a more recent study suggests that this change in side scatter is only temporary, and indeed was also seen in cells undergoing necrosis (Lizard et al(1995)). Indeed this same report commented upon the fact that apoptotic cells were unable to exclude Propidium Iodide (PI) at an earlier time point than had been previously realised, calling into question the use of these assays as a discriminant between apoptosis and necrosis. The same approach has been confirmed to discriminate necrotic from apoptotic cells only at an early stage (Bryson et al(1994)).

Most of the flow cytometric studies have used cells in culture, although at least one group has confirmed that the TUNEL end-labeling techniques do work in tumour FNAs, but there was no data on the ability to exclude necrotic cells (Gorczyca et al(1994)). In fact the proportion of apoptotic cells identified (1 - 51%) is far more than ever reported for morphological assessments, suggesting that the methodology employed could not differentiate between apoptotic and necrotic cells.

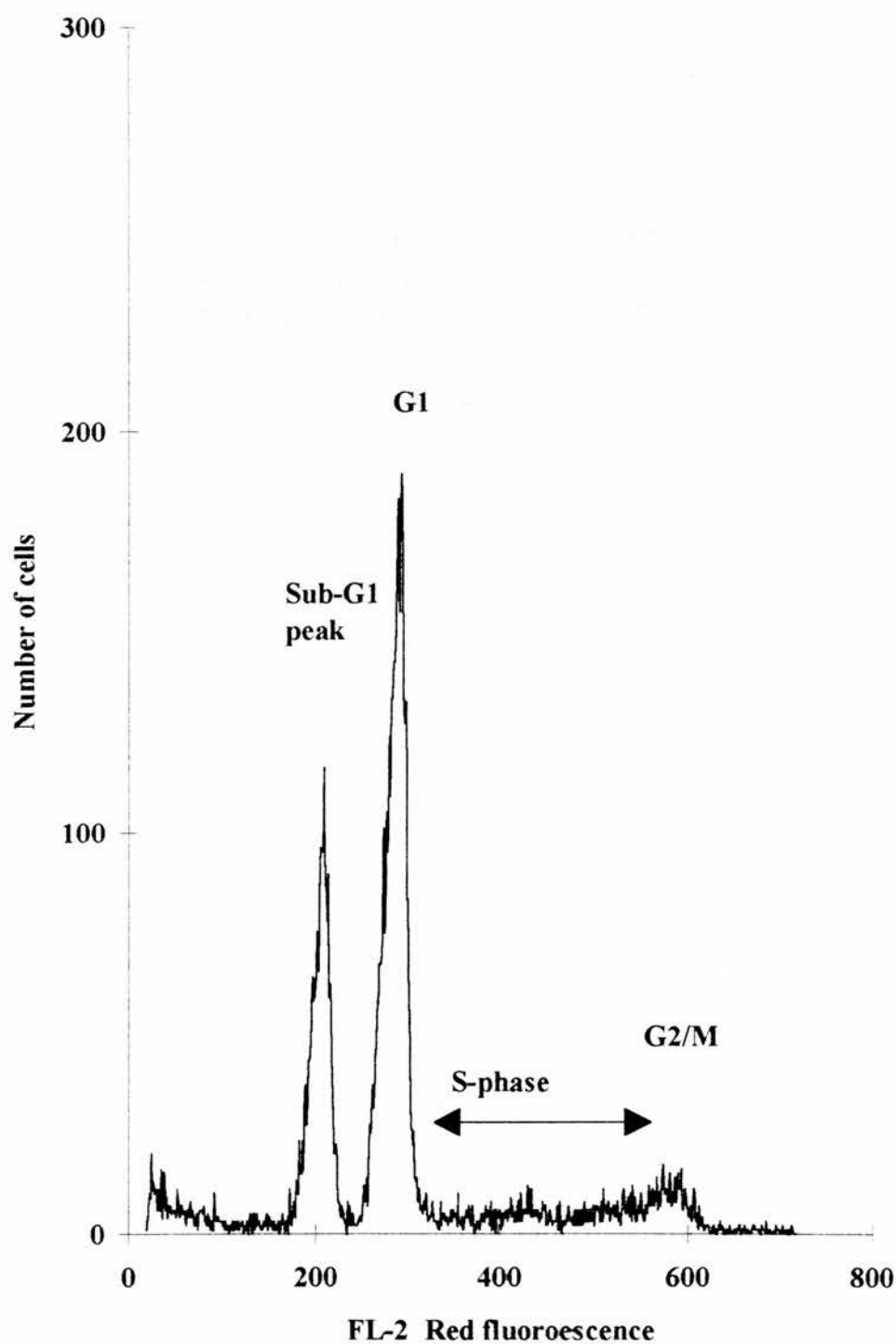


Figure 1 Flow cytometric analysis of DNA content (measured as red fluorescence) of a FNA taken from a ZR-75-1 breast cancer xenograft grown in a nude mouse, with a sub-G₁ peak containing cells derived from the host animal.

Bcl-2

Given the marked propensity of lymphoid cells to undergo apoptosis, it is perhaps of little surprise that the first gene to be associated with negative regulation of apoptosis was discovered to be overexpressed as a consequence of the t(14;18) translocation which appears to be pathogenic in the majority of cases of follicular lymphoma (Cleary et al(1985)). Named bcl-2, it was found to be widely expressed in many tissues and some tumours, including both benign and malignant breast (Nathan et al(1993)). The importance of this novel oncogene was not appreciated until it was demonstrated that it could inhibit the apoptosis which occurs upon serum-deprivation (Hockenbery et al(1990)). It is expressed in hormonally-sensitive glandular epithelial cells (Oltavi et al(1993)), often only in stem-cell and proliferation zones (Hockenbery et al(1991)), perhaps thus preserving the progenitor cells. There is considerable evidence for the apparent universality of the rôle that bcl-2 plays in inhibiting apoptosis, such as that induced by glucocorticoids and cytotoxics (Miyashita et al(1992)). The exact mechanism by which bcl-2 inhibits apoptosis remains unclear, but it appears to act down-stream of the initiation of apoptosis (Yin et al(1995)), and upstream of the activation of the I.C.E. cascade (Shimizu et al(1996)), possibly via the modulation of intranuclear Ca^{++} (Marin et al(1996)). Furthermore it has been reported to dimerise with another protein, bax, with which it has extensive homology (Oltavi et al(1993)). Overexpression of bax, which is capable of homo-dimerisation, accelerated apoptosis, whereas expression of bcl-2 resulted in hetero-dimerisation and reduced apoptosis. More recently it has been reported that bax is expressed in large amounts in the normal breast, to a lesser extent in MCF-7 and T47-D malignant breast cell lines (but not some others), and hardly at all in a small series of tumours (Bargou et al(1995)). Further members of the bcl-2 family have been identified including bak (Chittenden et al(1995); Kiefer et al(1995)), bad (Yang et al(1995)) and two forms of bcl-x : a long, apoptosis-inhibiting form bcl-x_L, and its inhibitor, the short bcl-x_S form (Boise et al(1993)).

However in breast cancer the rôle of bcl-2 is less clear. It is expressed in the majority of the breast cancers studied and is positively associated with ER status (Johnston et al(1994); Krajewski et al(1995); Lipponen et al(1995); van Slooten et al(1996)). It has even been suggested that bcl-2 expression was a better predictor of hormone-sensitivity than ER (Gee et al(1994)), and its expression was inversely related to both EGFR and c-erb-B2, putative markers of hormone insensitivity. Other studies have reported inverse correlations with poor prognostic features such as tumour grade, increased p53 expression, and c-erb-B2 over-expression (Hurlimann et al(1995); Joensuu et al(1994); Lipponen et al(1995); Sierra et al(1995); van Slooten et al(1996)) as well as increased proliferation as detected by either Ki-67 staining (van Slooten et al(1996)) or the S-phase fraction (Lipponen et al(1995); Steck et al(1996)). Bcl-2 positive tumours are more often well

differentiated, with low proliferation, and intact post-ER pathways, as evidenced by the expression of pS2 and cathepsin D (Hurlimann et al(1995)).

However the relationship between apoptosis and bcl-2 has not been fully elucidated in clinical breast cancer. Two reports of the inverse association of bcl-2 and apoptosis in breast cancers have used the indirect immunohistochemical methods of TUNEL (Sierra et al(1995)) or ISEL (Sierra et al(1996)) ; hence as discussed above, it is possible that the incidence of apoptosis was overestimated in tumours with significant necrosis. Furthermore the majority of grade III carcinomas did not express bcl-2, and high grade tumours are more likely to contain necrotic regions (Clayton, (1991)). Thus the observed inverse relationship between apoptosis and bcl-2 could in part be explained by the presence of necrosis in bcl-2 negative grade III tumours. There is a small study in 41 breast cancers that reported, particularly in tumours with a low mitotic rate, an inverse association between apoptosis, as measured by morphology, and bcl-2 expression (Chan et al(1993)). Finally another small study estimated the proportion of bcl-2 positive and apoptotic cells by flow cytometry, and again found evidence suggesting an inverse relationship (Steck et al(1996)). However apart from the potential problems of distinguishing between necrotic and apoptotic cells with the TUNEL technique, this study has the additional problem of potentially including non-malignant cells, some of which are known to express bcl-2 (Nathan et al(1993)). Hence the available data strongly suggest that bcl-2 expression is associated with reduced apoptosis, but is often based upon an indirect methodology, and analysis has not been restricted to ER positive (or bcl-2 positive) tumours.

Several studies have noted that bcl-2 expression is associated with a better survival for patients with early breast cancer (Hurlimann et al(1995); Joensuu et al(1994)). A large retrospective study reported that bcl-2 predicted for the "efficacy" of post-surgical adjuvant therapy (Gasparini et al(1995a)) ; however adjuvant therapy is administered to patients without any knowledge of their residual tumour burden. Thus a survival advantage for one group could be due to any combination of several reasons; more patients cured by surgery alone, more patients with slower growing tumours, or more patients whose disease is sensitive to the applied therapy. Thus to note a survival advantage for those given tamoxifen in the adjuvant setting could be because the tumours were more sensitive to tamoxifen - and the association between ER positivity and bcl-2 expression, as well as the data correlating response to tamoxifen and bcl-2 expression, both support the hypothesis that bcl-2 expression in the primary tumour of a patient given tamoxifen is likely to predict better survival.

The conclusions that can be drawn from the data in clinical breast cancer are that bcl-2 would appear to be associated with better differentiated tumours ; low grade, ER and PgR positivity, yet there is also the suggestion that bcl-2 inhibits apoptosis within these same tumours. However in cell systems,

highly proliferative cells, such as those overexpressing c-myc, are also more prone to undergo apoptosis. There are data to suggest that this may be the case in breast cancer (Lipponen et al(1994)) : although in that study both the mitotic and apoptotic indices were expressed per mm² of epithelium (corresponding to 5 x 400 high-power fields), a methodology that ignores variation in cellularity between different tumours. The same association has been reported in bladder and gastric carcinomas (Koshida et al(1997); Lipponen et al(1994))and lymphomas (Czader et al(1996)), suggesting that the in vitro observations may relate to the clinical setting. Hence it may be that bcl-2 is a marker of a particular breast cancer phenotype - low proliferation (and hence less apoptosis), low grade and more hormone sensitivity - and the expression of bcl-2 in normal breast epithelial cells would suggest that the persistent expression of bcl-2 is due to better differentiation. Consistent with this hypothesis is the data from breast cell lines where bcl-2 expression appears to be under oestrogen control (Teixeira et al(1995)), although this can be inhibited by tamoxifen (Wang et al(1995)). However a small clinical study would dispute this, finding that bcl-2 expression increased in 11 ER-positive tumours given tamoxifen before surgery (Johnston et al(1994)). The majority of these tumours were apparently negative for bcl-2 despite being ER positive, a finding that is not in keeping with other reported series, and in the absence of any data on response to tamoxifen, one cannot be sure whether these tumours are representative of the usual phenotype of ER-positive breast cancers.

p53, and p21^{WAF1}

p53

The oncogene p53 is a nuclear protein that has specific target DNA sequences, for which it acts as a transcriptional regulator (Tarunina et al(1993); Vogelstein et al(1992)). Mutations in this gene are amongst the most common genetic alteration observed in human malignancies (Hollstein et al(1991)). It appears to act as a "guardian to the genome", serving the very important function of inducing apoptosis in response to DNA damage (Lane, (1993)), such as that caused by ionising radiation or cytotoxics (Clarke et al(1993)). The cellular response to damaged DNA includes a rise in p53 levels, which in some cells causes G₁ arrest (Kastan et al(1992)), and in others apoptosis (Yonish-Rouach et al(1991)), predominantly in the G₁ phase of the cell cycle (Ryan et al(1993)). Mutant p53 appears to disable this cellular self-destruct pathway (Wang et al(1993)), which may thus render a cell less sensitive to cytotoxic therapy. Down-stream genes involved in p53-regulated events include mdm2 (Juven et al(1993)), p21^{WAF1} (El-Diery et al(1993)), bcl-2 and bax (Miyashita et al(1994); Selvakumaran et al(1994)). It has however recently been demonstrated that in response to the same stimulus p53 is capable of causing both growth arrest and apoptosis (Liebermann et al(1995)). This suggests that the outcome of p53 activation depends on other factors, including those which alter the propensity to undergo apoptosis. Consistent with this is the observation that for p53 to induce bax it requires the cell to be capable of undergoing apoptosis (Zhan et al(1994)). However as has been previously commented upon, apoptosis can occur independent of p53 activation. Evidence that this can occur in the same setting as p53-induced apoptosis comes from a study involving a cell line immortalized by c-myc and a temperature-sensitive p53 mutant (Sakamuro et al(1995)). With functioning p53, the cells underwent apoptosis, which was accompanied by G₁-arrest and increased expression of bax. In contrast, with the mutant p53, serum deprivation could still induce apoptosis, but without evidence of these other changes, although bcl-2 could suppress apoptosis with either form of p53.

The importance of p53 expression in clinical breast cancers remains under debate. Several studies have set out to determine the relationship between prognosis and overexpression, as detected immunohistochemically. Many of them have been reviewed recently (Mansour et al(1994)), with the conclusion that the majority of reports do find the presence of detectable p53 protein is a marker of poor outcome, particularly in node-negative breast cancer. Furthermore many studies report associations with other poor prognostic features ; high tumour grade (Cattoretti et al(1988b); Friedrichs et al(1993); Horne et al(1996)), lack of oestrogen receptors (Friedrichs et al(1993); Horne et al(1996); Thompson et al(1990)), increased proliferation (Cattoretti et al(1988b)) However the studies are not unanimous. One possible explanation is the use of different antibodies used for

immunohistochemistry ; indeed 40% of the tumours in a recent study gave inconsistent staining with four different antibodies each of which is in common use (Horne et al(1996)). There are fewer data relating p53 status with response to therapy. A small study of 17 patients treated with hormone therapy upon relapse reported that none of the 6 p53 positive patients responded (Horne et al(1996)) ; the ER status of these patients was not reported, and the majority of p53 positive tumours are ER negative (Archer et al(1995)). However, other studies have reported no relationship between p53 expression and hormone insensitivity (Archer et al(1995)). A large study sequenced the p53 gene in over 300 breast cancers and reported an association between p53 mutations and lack of response to adjuvant therapy (Bergh et al(1995)). However there is no absolute measure available for response to adjuvant therapy. Early disease progression and/or death may reflect lack of sensitivity to the applied therapy but equally could be a reflection of a more aggressive underlying biology. In contrast, patients remaining disease-free need not have benefitted from the adjuvant therapy, but rather have disease that is already cured or be more inherently indolent. Thus although the data are consistent with the notion that p53 mutations conferred resistance to both tamoxifen and perhaps CMF chemotherapy, it is equally possible that p53 mutations are associated with a poorer prognosis independent of the drug sensitivity of the disease. Consistent with this is the observation that p53 expression is associated with the presence of lymph node metastases (Vojtesek et al(1995)), the latter being consistently reported as the strongest prognostic indicator in early breast cancer (Bonadonna et al(1995); Bonadonna et al(1985); Botti et al(1995); Fisher et al(1983); Nemoto et al(1980); Schottenfeld et al(1976); Valagussa et al(1978)). Thus of greater interest is a small study of neo-adjuvant anthracycline-based chemotherapy in which it was reported that the presence of a particular p53 mutation conferred primary resistance to the chemotherapy (Aas et al(1996)). There are however no similar data available for tamoxifen.

p21^{WAF1/CIP1}

p21^{WAF1} is an inhibitor of the G₁ cyclin-dependent kinases involved in the G₁-S transition (Dulic et al(1994); Gu et al(1993)), including cyclin D1 (Harper et al(1993)). Although p21^{WAF1} has been implicated in p53-mediated growth arrest (El-Diery et al(1993)), it has a rôle to play in cellular differentiation that is unconnected with p53 expression. There is also evidence to suggest that p21^{WAF1} may be implicated in p53-independent apoptosis. Overexpression of bcl-2 in the MCF-10 breast cell line resulted not only in reduced apoptosis, but also reduced p21^{WAF1} expression (Upadhyay et al(1995)). In contrast in MCF-7 cells, overexpression of p21^{WAF1} caused giant cell formation and apoptosis (Sheikh et al(1995)). Furthermore tamoxifen induced growth arrest of both MCF-7 and T-47D cells is associated with increased expression of p21^{WAF1} (Maas et al(1995)), and since the latter cells do not have wild-type p53 (Vojtesek et al(1993)), this would suggest that it to be p53-independent.

Transforming growth factor- β

The family of type β transforming growth factors, TGF β_1 , TGF β_2 , TGF β_3 have been shown to be potent inhibitors of proliferation of most types of cells that have been studied (Alexandrow et al(1995)), although TGF β_1 has been the best studied member of the group. Cells are sensitive to this anti-proliferative effect in both early and late G₁, and certain cyclins and cyclin dependent kinases are their possible targets (Alexandrow et al(1995)and references therein). Furthermore TGF β can induce apoptosis in some cell systems, including uterine epithelial cells (Rotello et al(1991)), the prostate (Martikainen et al(1990)) and hepatocytes (Oberhammer et al(1992)). With respect to breast tissue, TGF β expression occurs in the mammary gland (Robinson et al(1991); Wakefield et al(1992)), and is growth inhibitory to normal human mammary epithelium (Hosobuchi et al(1989); Valverius et al(1989)). Malignant breast cancer cell lines however show a varied response to TGF β_1 , although growth inhibition occurs in the majority (Alexandrow et al(1995)). The data however on xenografts is scarce, and includes a report that no effect was seen when MDA-MB-231 tumours were treated with TGF β_1 (Zugmaier et al(1991)). Furthermore the TGF β s have profound effects on the stroma, largely stabilising the matrix (Alexandrow et al(1995)).

Angiogenesis

The limit of nutrient diffusion is around 1 mm, and thus for a tumour to grow beyond that size a separate blood supply is required (Folkman, (1990)). The development of these new vascular channels is termed angiogenesis, and it would seem that the process is driven by the tumour, with the host vasculature developing endothelial buds in response to growth factors secreted by the tumours. These buds eventually coalesce into a complete blood supply for the tumour. The importance of this angiogenesis has recently been elegantly confirmed by xenograft studies, where the volume of metastases was shown to increase significantly (as a consequence of reduced apoptosis) when the primary tumour was removed, an effect that could be inhibited by an anti-angiogenic factor (Holmgren et al(1995)). This would suggest that dormancy of tumour metastases is the consequence of impaired or inhibited angiogenesis (Folkman, (1995)), and there are data to suggest that one such anti-angiogenic factor, angiostatin, is produced by the primary tumour (O'Reilly et al(1994)). A large number of factors that both stimulate and inhibit angiogenesis have been reported, but amongst the inhibitory factors is tamoxifen (Gagliardi et al(1993)). There have been more than a dozen studies that have determined the prognostic relevance of the degree of angiogenesis in a patient's breast cancer (Gasparini et al(1995b)), and although their conclusions are varied, the majority report a survival detriment for women whose tumours have a high vascular density. There are methodological variations between the studies, both in the antibodies used to identify the vessels (usually either anti-factor VIII or anti-CD31), as well as the counting techniques. One of the more important studies found that in a group of over 250 patients with node-negative breast cancer, who had not received any adjuvant therapy, a high vascular density was independently associated with a worse outcome (Gasparini et al(1994b)). The exact relationship between vascular density and poor survival remains undefined, but the lack of any correlation with either mitotic/proliferative indices (Vartanian et al(1994)), and other poor prognostic markers such as p53, hormone receptor status, (Fox et al(1994); Gasparini et al(1994b)), suggests it is an independent factor rather than a marker of other poor-risk biological features.

Commercially, the available anti-endothelial cell antibodies are murine anti-human antibodies (to von Willebrand's factor VIII, CD-31 and CD-34). Thus none of these would be suitable for testing in a mouse tumour xenograft. We however had available a sample of a non-commercial antibody, MEC 13.3, which is a monoclonal rat anti-murine endothelial cell antibody (Vecchi et al(1994)), and works only on frozen tissue.

***In vitro* and *in vivo* effects of tamoxifen on breast cancer**

The rationale and clinical experience of tamoxifen therapy in breast cancer have already been discussed, and although the pharmacokinetics and pharmacodynamics have been well studied, they are not very relevant to this thesis. However one aspect does need to be mentioned, and that is that the major metabolite 4-hydroxytamoxifen is an active anti-oestrogen in its own right (Jordan et al(1977)), with a much higher binding affinity for the ER than its parent drug (Wakeling et al(1980)). Extensive laboratory studies with tamoxifen have determined much about the mode of action, and cell-cycle effects both *in vitro* and in model tumour systems. However the precise mechanism of action in patients is only partially understood, and in particular it remains unclear as to why some tumours manifest *de novo* resistance to the drug, despite having the hallmarks of hormone-sensitivity.

Experimental data

The initial model for tamoxifen's anti-oestrogenic action was as a competitive inhibitor of the ER by occupying the ligand-binding domain (Horwitz et al(1980)). This has been updated in the light of recent knowledge, particularly in view of the differing sensitivities of the two transcriptional activating regions, AF-1 & AF-2 (Parker, (1994)) ; it is proposed that the effect of tamoxifen binding to the ER is to not to prevent DNA-binding, but only to inhibit AF-2, whereas the activity of AF-1 remains unaffected. Thus tamoxifen (and its metabolites) can act as either an antagonist or agonist depending on the AF region of the receptor that is responsible for the particular oestrogen-induced effect. This therefore accords with the data suggesting tamoxifen can act as an agonist, particularly on the uterus (Wolf et al(1995)). There are however data to suggest that tamoxifen may have other mechanisms of action (reviewed in (Colletta et al(1994); Wolf et al(1995))). Protein kinase C (PKC) inhibition has been suggested as another mediator of tamoxifen's action (Wiseman, (1994)) , which could be important since PKC plays a key role in the transduction of membrane signals (Nishizuka, (1988)), and the PKC inhibitor H7 can induce apoptosis in lymphoid cells (Traganos et al(1993)). However any putative inhibition of PKC is unlikely to be the mechanism of tamoxifen's anti-proliferative actions, as PKC activators completely inhibit MCF-7 cell growth (Issandou et al(1990)), as does tamoxifen (Tominaga et al(1993)). Tamoxifen can also act as an anti-oxidant and alter cell membrane fluidity (discussed in (Vecchiotti et al(1980); Vogelzang, (1984); Wiseman, (1994))).

The anti-proliferative activity of tamoxifen has been well-documented. It was first demonstrated *in vitro* for the hormone-sensitive human breast cancer derived cell line, MCF-7; an effect that could be reversed by the addition of oestrogen (Lippman et al(1975)). Tamoxifen treated cells show evidence of G₁ arrest (Osborne et al(1983); Sutherland et al(1983a)), with an accompanying fall in the proportions of cells in S & G₂/M (Brunner et al(1989); Butler et al(1988); Danova et al(1993); Sutherland et al(1987)). Further characterisation of the mechanism of the G₁-block induced by ICI 164,384 and tamoxifen in MCF-7 cells suggests that rapid down-regulation of cyclin D1 may be crucial, since it occurred before any changes in entry to S-phase were detectable (Watts et al(1994)). Similar data have been reported for the pure anti-oestrogen ICI 164,384 administered to T-47D breast cancer cells (Musgrove et al(1993)). MCF-7 cells which had been xenografted into athymic mice also demonstrate growth inhibition by tamoxifen (Osborne et al(1985)), an effect which could be overcome by oestradiol (Iino et al(1991)). Therefore despite the initial suggestions that tamoxifen could be tumoricidal *in vitro* (Lippman et al(1975)), it was generally held that tamoxifen was a tumourostatic agent.

Data from *in vivo* experiments are less consistent, with some reports of decreased proliferation (Kute et al(1985); Osborne et al(1985)), and others finding evidence of increased cell loss or cytotoxicity (Brunner et al(1989)). One study has indeed found evidence of both mechanisms in MCF-7 xenografts (Sarkaria et al(1993)). However, as discussed above, cell loss occurs by two distinctive mechanisms, necrosis and apoptosis. Although apoptosis may be induced following oestrogen deprivation (Kyprianou et al(1991b); Szende et al(1989); Szende et al(1990); Warri et al(1993)), it is not a consistent finding, and a recent study reported no changes in apoptosis in ZR-75-1 xenografts following oestrogen-withdrawal (Kristensen et al(1995b)). Furthermore increased apoptosis has been observed with the tamoxifen analogue, toremifene (Warri et al(1993)), and with tamoxifen *in vitro* (Frankfurt, (1995); Perry et al(1995a)). However caution needs to be exercised in interpreting these reports, particularly those in which high concentrations of tamoxifen (as opposed to the more active metabolite 4-hydroxytamoxifen) have been employed *in vitro*; one study reported that tamoxifen could equally induce apoptosis in the ER-negative cell line MDA-MB-231 (Perry et al(1995a)), which was not found to occur with lower concentrations (Bardon et al(1987)), and may thus be a non-specific cytotoxicity (Sutherland et al(1983b)), perhaps mediated via TGF β , rather than the ER (Perry et al(1995b)). Interestingly, there are two reports that tamoxifen does not cause apoptosis in xenografts (Kristensen et al(1995b); Szende et al(1990)), but in both the mean apoptotic rate is higher with tamoxifen, although it is not statistically significantly so. In contrast, there are some data to suggest that tamoxifen can induce necrotic cell-death in xenografts (Haran et al(1994)) as a consequence of inhibiting angiogenesis (Gagliardi et al(1993)); but equally hypoxia has been reported to induce cell death that was termed apoptotic (Yao et al(1995)), although the techniques

employed (DNA ladder & TUNEL-staining) cannot alone distinguish the relative contributions of necrosis and apoptosis to the observed cell death. In conclusion therefore, tamoxifen may cause apoptosis in breast tumours, although the data are not completely consistent, particularly with regard to the ER-specificity of the process.

Given the known anti-proliferative effects of the TGF- β s (reviewed in (Alexandrow et al(1995); Wakefield et al(1992))), they are possible effectors of tamoxifen's actions, and have been thus studied. Their expression is under hormonal control, being inhibited by oestrogens (Dickson et al(1986)) and stimulated by tamoxifen (Knabbe et al(1987)), although not in ER negative cell lines. However the net effect of the combination of oestrogens and anti-oestrogens appears to be critically dependent on the experimental conditions (Wakefield et al(1992)). There is evidence that tamoxifen can increase TGF β_1 activity in association with, and indeed before the onset of, apoptosis (Chen et al(1996); Perry et al(1995b)). Perry's study employed 10 μ M tamoxifen (Perry et al(1995b)) and confirmed the effect on G₁ block, but reported that the induction of apoptosis occurred in both ER positive and ER negative cell lines. However no increase in apoptosis was seen upon oestrogen withdrawal, which conflicts with previous reports (Szende et al(1989); Szende et al(1990); Warri et al(1993)), including Kyprianou's work which documented a rise in TGF β in ER positive MCF-7 xenografts as they regressed consequent upon oophorectomy (Kyprianou et al(1991b)). Perry's study raises the possibility of tamoxifen having an effect upon TGF β levels independent of the oestrogen receptor, and consistent with this is the report that it can induce TGF β secretion from human foetal fibroblasts (Colletta et al(1990)).

Clinical data

In clinical breast cancers there have been few reports of the biological effect of tamoxifen therapy, and only one that sought to identify changes that parallel or even predate tumour response (Keen et al(1997)). There are data to suggest that tamoxifen can reduce proliferation (as assessed by Ki-67) irrespective of the ER status of the tumour (Clarke et al(1993)). A more recent study reported no effect on ER-negative tumours but a 30% reduction in proliferation in ER-positive tumours, although this was not statistically significant even at the 10% level (Dowsett et al(1995)). The investigation however suffered from the defect that only indirect comparisons could be made as the control values were obtained from untreated tumours in age-matched patients. Apoptosis was also assessed in the same tamoxifen treated tumours by the ISEL method (Wijsman et al(1993)), and the apoptotic index of most tumours was found to be below an arbitrary 1% threshold. A trend ($p = 0.08$) towards more apoptosis in treated ER-positive tumours was however reported, with a significantly less apoptosis in ER-positive tumours as compared to ER-negative tumours, that paralleled a similar difference in proliferation. Furthermore the proliferation:apoptosis ratio was significantly less only in the ER-

positive treated tumours. However these studies examined tumours after 6 - 65 and 5 - 35 days' treatment respectively, and thus could not comment on the relationship between any changes and tumour response. In contrast, the study of Keen et al (Keen et al(1997)) reported significant associations between tumour response after 3 months' tamoxifen, and falls in proliferation (as assessed by Ki-S1) and bcl-2 expression (which might thus reflect an increase in propensity to apoptosis). It is on this same set of tumours that the current study will assess changes in mitosis and apoptosis. An obvious candidate for examination of the effect of tamoxifen would be the product of any gene known to be under oestrogenic control. The Oestrogen and Progesterone receptors as well as the protein pS2 could thus all be potential markers of true endocrine-sensitivity, but the few studies reported find no consistent relationship between changes in expression and response. A fall in the level of ER has been reported, with no significant change in either pS2 or PgR (Johnston et al(1995)), but no response data were available. In contrast, another study reported decreases in both ER and PgR, and a significant rise in pS2 but no change in c-erb-B2 (Soubeyran et al(1996b)). However none of the observed changes correlated with response to 5 months' tamoxifen therapy.

As discussed earlier (page 33), the TGF β family are potential mediators of certain tamoxifen effects. However the data from clinical studies do not confirm changes in their levels of expression to be strongly associated with tamoxifen sensitivity. Butta (Butta et al(1992)) demonstrated an increase in extra-cellular TGF β_1 (but not TGF β_2 or TGF β_3) following three months' therapy with tamoxifen. This effect was however noted to occur in both ER negative and ER positive tumours, and no association with tumour response was reported. In contrast, MacCallum reported that the same duration of therapy, in women with ER positive tumours, was not associated with any consistent change in mRNA levels of any of the three members, but that there was a significant increase in TGF β_2 levels in responding tumours (MacCallum et al(1996)). Finally in advanced disease there is a report that TGF β_1 can be cleaved to a smaller 50kDa protein whose expression is associated with tamoxifen resistance (Baillie et al(1996)), consistent with reports that the over-expression of TGF β_1 can induce oestrogen-independence in MCF-7 xenografts (Arteaga et al(1993)). Hence this family of proteins was not considered further in this study.

Assessment of response

In considering the efficacy of an anti-cancer therapy, one has to define the purpose of that treatment. In the long term, the patient's interest is usually best served by cure of the disease, but for many patients that is an unachievable goal. The assessment of a therapeutic strategy cannot always await the long-term survival of patients, and in the arena of palliative treatments, other short-term gains such as improvement of tumour-related symptoms are sought. Therefore tumour regression often becomes an end-point in itself, and the precise measurement of this can be difficult. Primary breast cancer affords the possibility of a more direct tumour volume measurement than is often possible for later stage disease, but still has the problem that within the malignant tumour there are benign structures such as stroma, infiltrating lymphocytes, blood vessels and fat. Furthermore clinical tumour measurements are prone to error (Warr et al(1984)), and not just because of the thickness of skin and sub-cutaneous tissue. Radiological measurements of primary breast cancers offer an alternative method of sequential tumour volume measurements. Mammography can be helpful, but is uncomfortable for the patient, and does not identify a distinct tumour volume in all cases. An alternative is ultrasound, which is painless and does not require the same expertise as mammography. This technique, although it has been shown to be more accurate than clinical measurements (Forouhi et al(1994)), still tends to overestimate as compared with the true pathological volume (Nishimura et al(1988)). However any clinical measure of tumour volume is static, and does not take into account the biological mechanisms of tumour response.

It is clear that tumour growth or regression is a consequence of the balance between cell proliferation and cell death (Schwartzman et al(1993)) and any alteration in this balance will change the growth curve of the tumour. It remains unclear however what are the relative contributions of apoptosis and necrosis to the overall cell loss. Furthermore in assessing tumour response (or otherwise) to therapy, no account is taken of what cellular changes occur: the most widely accepted system for defining response, that proposed by the UICC, defines a response as a fall of at least 50% in the product of two orthogonal diameters of a tumour, with a complete response occurring when no clinically detected tumour remains (Hayward et al(1977); Miller et al(1981)). However if one considers the possibility of tumours with different proliferation rates, it is immediately apparent that the same clinical response can occur as a result of different biological responses in the tumour. Assume, for example, that the average cell cycle length is 24 hours, and the lifespan of a morphologically apoptotic cell be 6 hours (consistent with (Berges et al(1993))). Consider then two tumours with the same apoptotic:mitotic ratio; Tumour A, is a slow growing tumour with a proliferative fraction of 1% and an incidence of apoptotic cells of 0.125%. In one day, 1% of the cells divide, and 0.5% of the cells

die by apoptosis. Hence the growth rate is 0.5% per day, giving a doubling time of 139 days. In contrast tumour B, which has a higher proliferative fraction of 10%, and an apoptotic cell incidence of 1.25% will grow at 5% a day, and double its volume in 15 days. Thus to induce a 50% reduction in tumour volume over any given time period, the increase in cell loss rate has to be much higher in the slower growing tumour. Hence an anti-proliferative drug that reduces proliferation by 75% will induce a response in the fast tumour in 28 days, but not until 284 days in the slow tumour, assuming no resistance develops over the 9 months of therapy! Conversely, for a pure cytotoxic (i.e. a drug with no anti-proliferative effect) to induce a response by three months (i.e. 90 days), the increase in cell kill for the fast tumour needs only to be 116%, whereas in the slow tumour it is more than twice as great at 255%. However at the same time, the potential tumour growth saved by the drug administration is much greater in the faster tumour with over a hundred-fold reduction from an untreated volume (assuming no decelerating growth) in contrast to the 70% reduction for the slower tumour (see figure 2, noting the log-scale) ! Recalling that most cytotoxics are more effective in faster proliferating tumours, one can immediately see that a more powerful drug is required to demonstrate the same efficacy (by UICC criteria) in a slower-growing tumour.

Thus for a sensitive analysis of how a tumour responds to tamoxifen, it would be beneficial to know its growth rate, which is usually not possible. An alternative then is to change the definition of response to a continuous variable, being the percentage volume reduction. This does not avoid all pitfalls, but permits lesser degrees of response to be considered.

Xenografted tumours do however afford the possibility of observing the pattern of growth before therapy starts, as well as the continued growth in the untreated animals. Deducing future potential growth can then be done by fitting a curve to the initial volumes and extrapolating, with the tumour response then defined no longer as a reduction from the volume at the initiation of treatment (which will be called the relative volume), but from the potential untreated volume (see figure 2), noting that both tumours have the same volume before and after treatment). This concept, of using predicted growth as the basis on which to assess treatment efficacy has been proposed (Norton et al(1976)), but not to my knowledge ever used.

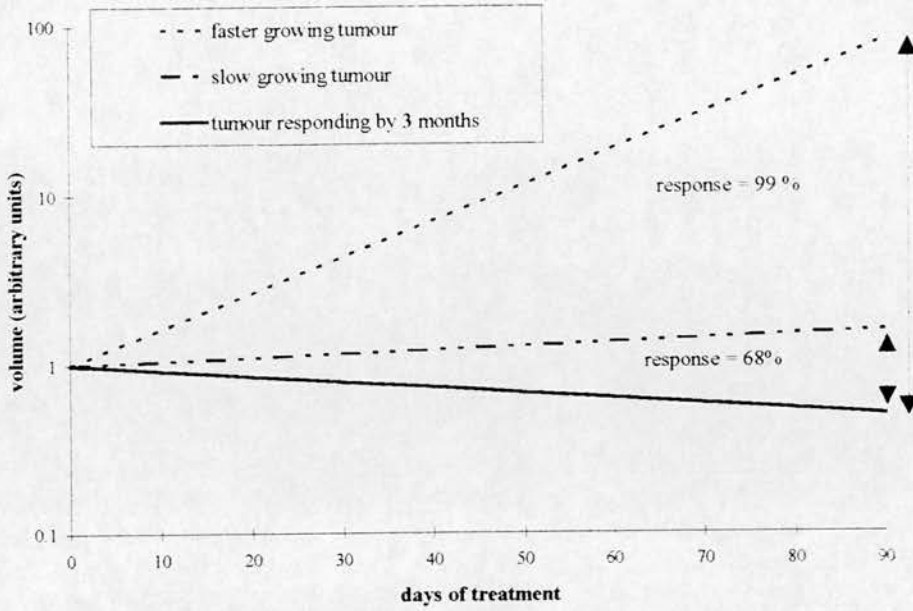


Figure 2 Effect of tumour growth rate on response to therapy

It remains therefore to consider the methodology for fitting growth curves to the xenograft volume data. There is a large body of work on fitting different growth curves to experimental tumour growth, but no better model than that originally described by Gompertz (Gompertz, (1825)) has been found to describe the decelerating growth that is clearly seen when malignant cells are grown in spheroids (Marusic et al(1994a); Marusic et al(1993)) or as xenografts (Durbin et al(1967); Laird, (1965); Laird, (1964); Marusic et al(1994b); Simpson-Herren et al(1970); Spang-Thomsen et al(1980)) particularly when the data span a wide range of volumes (Steel, (1977)). Mathematically this describes exponential growth, but the growth rate decays exponentially with time (and thus volume) such that there is a finite maximum volume, to which the growth curve is asymptotic (see figure 3). This is expressed as :

$$V(t) = V_0 e^{(\frac{\alpha}{\beta})(1-e^{-\beta t})} \quad (I)$$

where $V(t)$ is the tumour volume at time t , V_0 the volume at time $t = 0$, and α and β are parameters describing the initial growth rate (α/β) and the rate of decay from exponential growth (β). The theoretical maximum volume, when $t = \infty$, is equal to $V_0 e^{\alpha/\beta}$. It has been noted that this theoretical maximum volume appears to be roughly constant for a given species (Brunton et al(1978)). The acceptance of this model has been further increased since it has been found to describe clinical tumour growth of myeloma, (Sullivan et al(1972)) breast cancers (Norton, (1988)) and lung

metastases from testicular tumours (Demicheli, (1980)). However it should be noted that other workers have found that exponential growth (Brown et al(1984)), logistic models (Spratt et al(1993)) or intermittent Gompertzian growth (Speer et al(1984)) have also fitted clinical data well, and that in many cases the Gompertz curve has been fitted to very few data points, which could equally fit exponential or other functions (Cameron, (1997)).

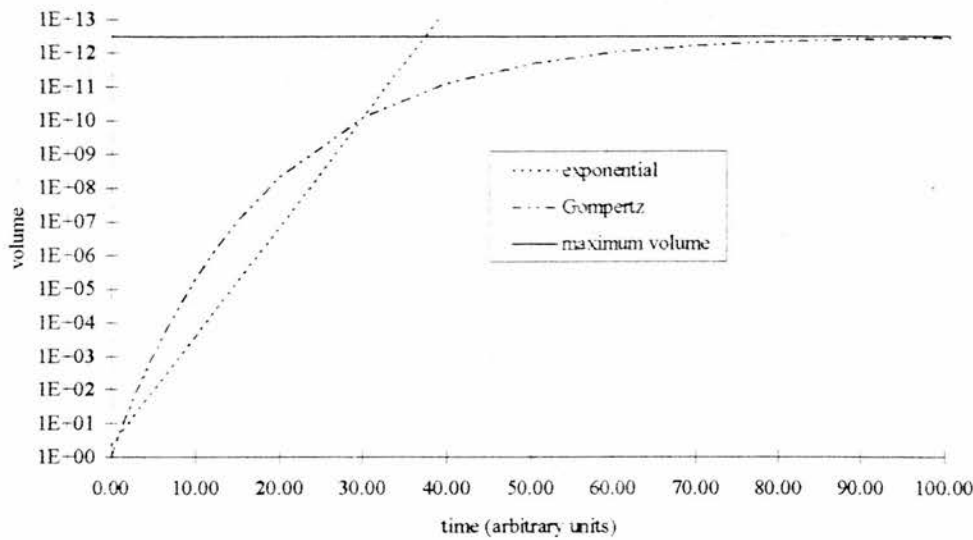


Figure 3 Exponential and Gompertz growth curves

For the prediction of xenograft tumour volumes the Gompertz model will be used, but with one small modification ; since the initial tumour volume (V_0) is unknown, a third variable (τ) is required, being the time difference from when the volume was 1 to when it was first clinically apparent, thus eliminating the unknown V_0 :

$$V(t) = e^{(\frac{\alpha}{\beta})(1-e^{-\beta(t+\tau)})} \tag{II}$$

Having defined the equation to which the tumour volumes must fit, the next issue is to choose the methodology, as this is not a linear function. In the literature cited above, two options have been used. The most definitive comparison of different growth models as applied to tumour spheroids noted that not only was the Gompertz function as good as any other, but also that this was best done

by using the method of least squares fitted to the logarithms of the tumour volumes rather than the volumes themselves (Marusic et al(1994b)). Mathematically equation (II) then becomes :

$$\ln(V) = \frac{\alpha}{\beta} \left(1 - e^{-\beta t + \tau} \right) \quad (III)$$

and the curve-fitting task is to minimise :

$$\sum_{i=1}^n \left(\ln V_i - \frac{\alpha}{\beta} \left(1 - e^{-\beta(t + \tau)} \right) \right)^2$$

where V_i are the actual measured volumes.

In order to do this complicated mathematical routines are required, and advantage was therefore taken of a general modelling package available to run on micro-computers under DOS, called ADAPT (D'Argenio et al(1990)) which allows the actual function (i.e. equation III) to be user-specified.

However it should be noted that there is an alternative methodology, as used in one study fitting Gompertz function to individual xenograft tumour data (Spang-Thomsen et al(1980)). That is if the natural logarithm is twice taken on both sides of equation I, one has :

$$\begin{aligned} \ln \left(\frac{\alpha}{\beta} - \ln(V_i) \right) &= \ln \left(\frac{\alpha}{\beta} \right) - \beta t \\ \Rightarrow \ln \left(\ln \left(\frac{V_{\infty}}{V_i} \right) \right) &= \ln \left(\frac{\alpha}{\beta} \right) - \beta t \end{aligned} \quad (IV)$$

as $e^{\alpha/\beta}$ is the same as V_{∞} . Thus by using a known or estimated value for V_{∞} , fitting the Gompertz curve becomes a simple linear problem, and this can be achieved by performing linear regression of $\log [V_{\infty}/V_i]$ on the time t . The disadvantage of this method is that it does require an accurate estimate of the maximum volume, or alternatively a two-step minimization procedure : firstly to estimate the maximum volume for that individual tumour and then to use linear regression to obtain values for α and β . Computationally this can be done using the Microsoft programme Excel, by

minimising the deviation of equation IV from linearity as defined by the coefficient for the correlation between the values of $\ln[\ln[V_{\infty}/V_i]]$ and t_i , the time points when the measurements were made. But if the tumour volume data suggests an unexpectedly high or low maximum volume as a consequence perhaps of errors in a small number of measurements, the resulting curve may be unreliable or even not definable. The approach using the non-linear equation III does not suffer from this potential problem, as all three parameters α , β and τ are estimated concurrently, and has therefore been used in this study.

Materials & Methods

Study design

Clinical study

To use a series of 50 pairs of tumour tissue taken before and after three months' tamoxifen therapy from patients with histologically proven ER-positive invasive breast cancer. The level of expression of bcl-2 (a cell survival gene and thus a potential surrogate marker for apoptosis), had been previously determined in these tumours, and both the absolute level and tamoxifen-induced change in expression have been reported to correlate with clinical response to tamoxifen therapy (Keen et al(1997)). Further sections from the same tumour blocks were cut, and after routine H & E staining, examined for mitosis and apoptosis. The apoptotic and mitotic indices were then compared with the expression (and changes in expression) of bcl-2 in order to ascertain whether the previously noted changes in this surrogate marker did indeed reflect appropriate changes in biological processes. Furthermore the changes in apoptotic and mitotic indices were analysed, in conjunction with the changes in bcl-2 and ER, in order to determine the effect of tamoxifen upon tumour biology.

Model xenograft study

To determine the chronology of the tamoxifen-induced changes in apoptosis & mitosis and their relative timing as compared with tumour response in a model system, comprising xenografts of the hormone-dependent (ZR-75-1) and hormone-independent (MDA-MB-231) breast cancer cell lines. The advantage of a model system was that precise growth and regression curves could be obtained, and correlated with any observed changes in cell death or proliferation. Multiple invasive techniques could be employed and control groups of untreated and tamoxifen-insensitive tumours used for comparison. Given that this model system permitted more precise estimation of the timing of any changes, it also afforded the opportunity to examine changes in other markers that might reflect the biological changes consequent upon anti-oestrogen therapy. Finally the use of FNA samples was assessed in parallel to determine whether that technique held any promise for enabling early in vivo prediction of tamoxifen sensitivity whilst obviating the need for a second surgical intervention.

Materials

Clinical breast cancers

These comprised tissue taken before and after tamoxifen therapy from 50 elderly women, attending the Edinburgh Breast Unit, to whom the drug had been administered for three months prior to definitive loco-regional surgery. The women are usually over 70 years old, and have a primary breast cancer of at least 3cm in maximum diameter, and in whom there were no obvious metastases by routine staging tests. Many have operable disease - for others it is locally inoperable because of fixed axillary nodes (N_2 disease) or skin or muscle involvement (T_4 disease). The purpose of pre-operative tamoxifen therapy is to attempt surgical downstaging, and to establish *in vivo* tamoxifen sensitivity. Thus pre-operative tamoxifen therapy is only offered if the tumour ER concentration is at least 20 fmol/mg cytosolic protein. ER measurement and histological confirmation of invasive breast cancer are established on a pre-treatment tumour biopsy, from which additional material is usually made available for research. After 3 months' tamoxifen therapy, provided there has been no tumour progression, the patients are then offered the option of electing to continue with tamoxifen alone, to undergo definitive loco-regional surgery, or if they are uncertain of their own preferences, to enter a trial which randomises them between these two options. Over a period of 2 years, 50 patients underwent pre-operative biopsy, 3 months' tamoxifen and subsequent loco-regional surgery, and thus from whom tissue was available both before and after tamoxifen treatment. During the tamoxifen therapy, the patients' tumours were monitored clinically and by ultrasound at four weekly intervals. Tumour response to tamoxifen was calculated as the percentage change in volume over the three months' therapy. At surgery tumour tissue was snap frozen in liquid N_2 , subsequently thawed, fixed in formalin for at least 24 hours and mounted in paraffin-wax blocks, from which serial sections were cut.

Xenografts

The oestrogen-sensitive ZR-75-1 breast cancer cell line, and the oestrogen-insensitive MDA-MB-231 breast cancer cell line, were maintained by sequential passage in female nude athymic mice (HsdOla:ICRF-nu), purchased from Harlan (UK), BICESTER, Oxon. At each passage a 1 mm fragment of tumour was inserted subcutaneously in each flank of the animal when the mice were 8 - 12 weeks old ; on the same day a slow-release oestrogen pellet (0.72 mg, released over 60 days supplied by Innovative Research of America, Ohio, U.S.A.) was inserted subcutaneously in the midline. All mice were maintained on a standard diet (Harlan (U.K.)), and water ad libitum. Bidimensional tumour diameters were recorded, and tumour volumes were calculated as

$$\text{Volume} = \pi/6 \cdot D \cdot d^2, \text{ where } D \text{ was the larger of the two diameters.}$$

Once the average of the tumour volumes reached about 0.25 cm^3 , the animals were randomly assigned to either a treatment group in whom a slow-release tamoxifen pellet (2.5 mg over 60 days, Innovative Research of America) was inserted sub-cutaneously adjacent to the oestrogen pellet ; or a control group in whom no further pellet inserted. There were no treatment-related animal deaths.

Fine Needle Aspirates (FNAs), were performed using a sterile 5ml Plastipak syringe and a 23G Yale Microlance needle, with a minimum of 10 passes made through the tumour, whilst the animal was restrained manually. 2 ml of standard freezing mixture (10% DMSO and 90% foetal calf serum) was then aspirated in to the syringe. The contents were snap frozen in liquid N_2 , and transferred to -80°C for storage. If the animal was to only have one of two tumours excised, the FNA was performed after the administration of the anaesthetic, in order to minimise the time interval between FNA and tumour excision.

For animals bearing single tumours, fine-needle aspiration was performed immediately prior to the animal being killed by cervical dislocation, following which the tumour was excised. Animals bearing two tumours were anaesthetised using hypnorm and hypnovel, and the tumour subjected to a FNA before excision (the surgical wound being closed with MICHEL clips). The remaining tumour was excised at the later time-point in the same manner as for animals bearing one xenograft. Excised tumours were immediately snap frozen in liquid N_2 , and then transferred to -80°C for storage.

Response to treatment

Two measures of response were considered. The first, termed "relative volume" was the ratio of the volume on treatment to that at the start of treatment. The second, called "response", was the proportional reduction from the predicted volume at the same time point, using an extrapolated gompertz growth curve fitted to that tumour's pre-treatment volumes. Thus a response of 0 implies the treated volume was identical to the predicted volume, whereas a response of 1 occurred when the treated tumour volume was 0. A negative response implied that the treated volume was larger than that predicted by the growth curve. This particular definition was chosen such that the values would be similarly interpreted as for the clinical tumour response, although the latter was based on the actual pre-treatment volume rather than a predicted untreated volume.

Study 1 : animals bearing either ZR-75-1 or MDA-MB-231 tumours

ZR-75-1 tumours were used from three passages, numbers 25, 28 and 29, as illustrated in table 1. In the first passage, 25 animals were established with ZR-75-1 tumours in both flanks, and all animals were administered tamoxifen after the day 0 tumours had been excised. One tumour in every animal was left to grow until day 28 : 5 animals had one tumour removed on each of days 0, 2, 7, 14, and 21. However on day 28, one of the tumours could not be palpated, giving rise to a total of 49 tumours from that experiment. Subsequently, from passage 28, 7 animals were established with xenografts, (3 were single and 4 double takes). In one animal both tumours regressed before day 0, and are thus not included in the results. None of these animals were given tamoxifen : 3 tumours were removed on day 0, leaving 6 to be removed on day 28. Finally (passage 29) 10 animals were established with two tumours each, and a further 16 with single tumours. The protocol for tumour removal in passage 29 was more complicated, in order to have roughly equal numbers of tumours at the various time-points. Amongst the untreated animals, the one animal bearing two tumours had one each removed on day 0 & 28, whereas the two animals with single tumours both had them removed on day 28. In the remaining 23 animals allocated to the treatment group, 3 mice with double tumours had one removed on day 0 and another on day 28. The remaining mice had tumours removed on either days 2 & 14, or days 7 & 21, apart from six mice bearing single tumours of which 3 each were removed on days 14 & 21.

| days of tamoxifen | 0 | 2 | 7 | 14 | 21 | 28 | day 28 control |
|---|-------------|-------------|-------------|-------------|-------------|----------------------|----------------|
| passage 25 | -----5----- | -----5----- | -----5----- | -----5----- | -----4----- | -----4----- | |
| | | | | | -----1----- | | |
| passage 28 | -----3----- | | | | | | -----3----- |
| | | | | | | | -----3----- |
| passage 29 | -----4----- | | | | | | |
| | | -----4----- | | | | | |
| | | | -----3----- | | | | |
| | | | | -----3----- | | | |
| | | | | | -----3----- | | |
| | -----3----- | | | | | -----3----- | |
| | | -----3----- | | -----3----- | | | |
| | | | -----3----- | | -----3----- | | |
| | | | | | | -----4 (3 mice)----- | |
| Totals | | | | | | | |
| Treated | | 12 | 12 | 11 | 11 | 27 | |
| Control | 11 | | | | | | 10 |
| -----4-----4 indicates 4 tumours removed at each of the two time points, all from mice bearing two tumours. | | | | | | | |
| -----3-----3 indicates 3 tumours removed at one time point from mice bearing single tumours. | | | | | | | |

Table 1 Details of timing of tumour removal for ZR-75-1 xenografts

MDA-MB-231 tumours from passages 2 & 7 were also established in a similar manner, as shown in table 2. At passage 2, there were 6 mice bearing two tumours allocated to the treatment group, with three tumours removed as day 0 controls, and three having their first tumour removed on day 14. The intention had been to remove all six remaining tumours until day 28, but two mice were sacrificed prematurely because of tumour ulceration. Four "single take" mice were allocated to the treatment group and had their tumours removed on day 7, and 6 mice with "single takes" left untreated; four had their tumours removed on day 28, and the other two on day 0. In the subsequent experiment, passage 7, there were 13 "double takes" and 17 "single takes". However because of faster growth in this experiment than the previous one, the experiment had to be terminated on day 21. 3 mice bearing one tumour and one mouse bearing two tumours were left untreated, and all four of these control tumours were removed on day 21. A further 5 mice had their only tumour removed on day 0. In the treatment group, one mouse bearing two tumours had one tumour removed on day 0, with the

other on day 21. 6 treated tumours were removed on both day 2 and day 7 - three from mice with single tumours, and three from mice with double tumours, whose second tumour was removed on day 21. On day 14 three tumours from "double take" mice and two from "single take" mice were removed, but one animal in this group had to be sacrificed prematurely because of tumour ulceration, leaving only two tumours to be removed on day 21. Finally 3 "single take" and 2 "double take" mice had their tumours treated until day 21 when all were removed.

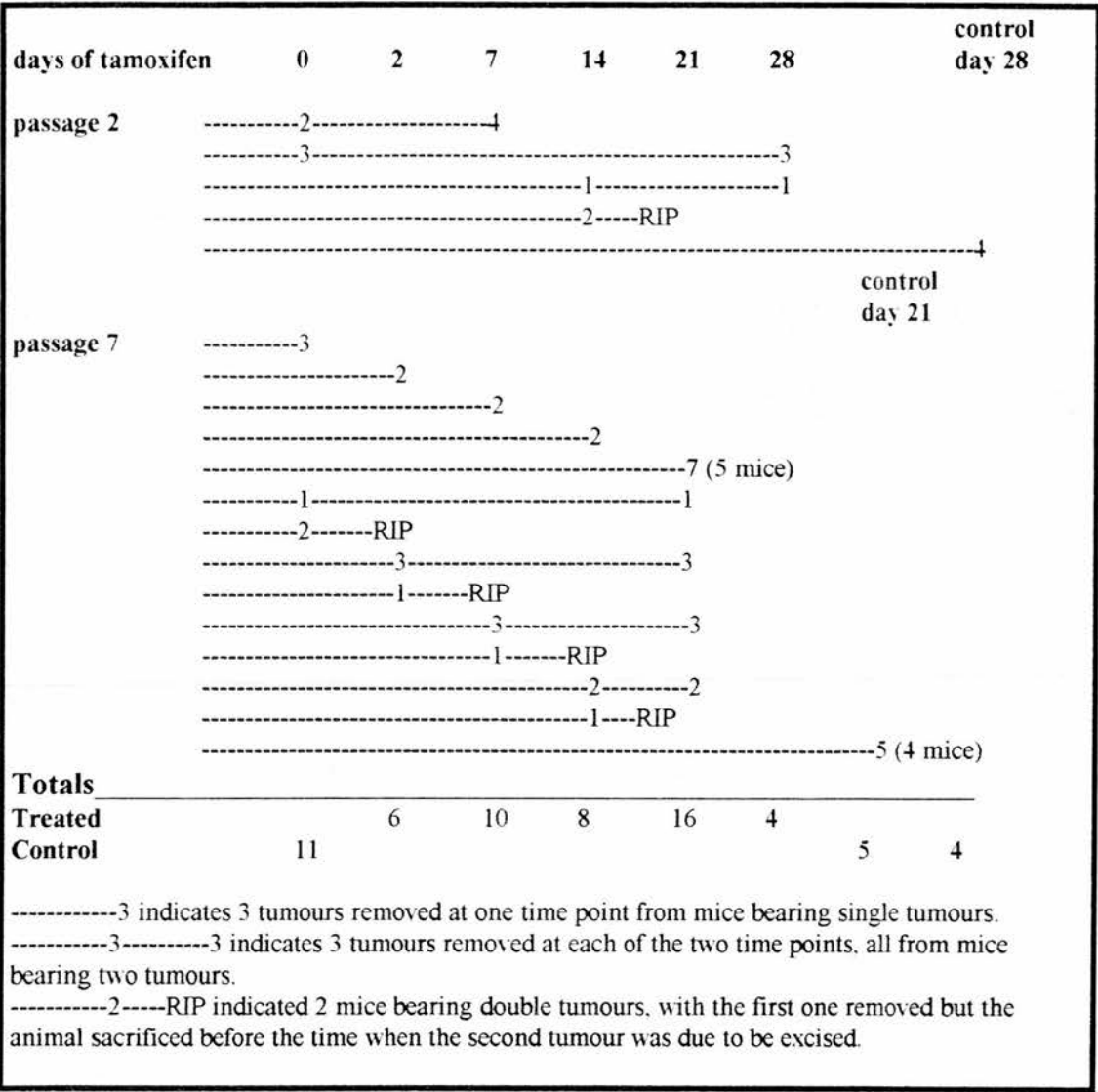


Table 2 Details of timing of tumour removal for MDA-MB-231 xenografts



Study 2a : mice bearing synchronous ZR-75-1 and MDA-MB-231 tumours

Animals had a 1 mm fragment of ZR-75-1 tumours (passage 36) implanted in their left flank, and a 1mm fragment of MDA-MB-231 (passage 9) implanted in their right flank. Of the 48 animals, both tumours took in 24 animals, only ZR-75-1 tumours grew in 12 animals, and only MDA-MB-231 tumours grew in a further 12. The animals with two tumours were managed as follows : 4 were used as day 0 controls with all tumours being removed on day 0, 10 each were either administered tamoxifen or left untreated, 5 animals having both tumours removed on day 2, and the remaining 5 having both tumours removed on day 7.

Excised tumours

| | | | |
|--------------------------|----------|----------|----------|
| days of tamoxifen | 0 | 2 | 7 |
| treated | | 5 | 5 |
| control | 4 | 5 | 5 |

numbers indicate the number of mice killed at each time point ; each mouse bearing one ZR-75-1 tumour and one MDA-MB-231 tumour.

Table 3 Details of animals bearing synchronous MDA-MB-231 & ZR-75-1 xenografts

Study 2b : mice bearing single tumours subjected to sequential Fine needle aspiration

The remaining 12 animals bearing single tumours of each type (ZR-75-1 or MDA-MB-231) were evenly split between treatment and control groups, and had FNAs performed on days 0,2,5,7,9,12,14, and 21, with the tumours excised on day 21; although one mouse bearing a MDA-MB-231 tumour died on day 5.

| days of tamoxifen | 0 | 2 | 5 | 7 | 9 | 12 | 14 | 21 |
|---|---|---|---|---|---|----|----|----|
| treated | | | | | | | | |
| ZR-75-1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MDA-MB-231 | 6 | 6 | 5 | 5 | 5 | 5 | 5 | 5 |
| control | | | | | | | | |
| ZR-75-1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| MDA-MB-231 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| numbers indicate the number of mice whose tumours were subjected to an FNA. | | | | | | | | |

Table 4 Details of animals bearing synchronous MDA-MB-231 & ZR-75-1 xenografts subjected to sequential FNAs

Immunohistochemistry

Antibodies

| | Microwave | Dilution | Incubation | Supplier |
|--------------------------------------|-----------|----------|----------------|--|
| primary | | | | |
| bcl-2 | yes | 1:100 | 4°C, 16 hours | DAKO. |
| c-erbB-2 | no | 1:40 | 20°C, 1 hour | NOVOCASTRA. |
| MIB-1 | yes | 1:50 | 20°C, 1 hour | IMMUNOTECH S.A.. |
| p53 (DO7) | yes | 1:30 | 20°C, 1 hour | NOVOCASTRA. |
| MEC 13.3 (endothelial) | no | 1:2 | 20°C, 1/2 hour | Prof. Mantovani, Istituto Mario Negri, MILAN |
| secondary | | | | |
| biotinylated rabbit anti-mouse | | 1:100 | 20°C, 1/2 hour | DAKO. |
| fluorescein-conjugated goat anti-rat | | 1:20 | 20°C, 1/2 hour | CALTAG. |

| Reagents | Supplier |
|---|--------------------------|
| ABC complex | DAKO |
| Alcohol | FISONS |
| Citric Acid | SIGMA |
| DMSO (dimethylsulfoxide) | NBS biologicals |
| DAB (diaminobenzidine) | SIGMA |
| DPX mountant | FISONS |
| Eosin | SIGMA |
| Foetal calf serum | GIBCO |
| Haematoxylin | SIGMA |
| Hydrogen peroxide | SIGMA |
| Lysine coated slides | SIGMA |
| NaCl | SIGMA |
| NaOH | SIGMA |
| Paraformaldehyde | SIGMA |
| PBS (Phosphate buffered Saline) | SIGMA |
| TBS (0.05M Tris-buffered Saline pH 7.5) | SIGMA |
| Trypsin (1 in 250) | DIFCO |
| Xylene | FISONS |
| Flow Cytometry | |
| DNAase I | SIGMA |
| Nonidet P40 | SIGMA |
| PBS (Phosphate buffered saline) | SIGMA |
| Propidium Iodide | FLUKA |
| RNAase A | SIGMA |
| Spermine tetrahydrochloride | SIGMA |
| Sucrose | SIGMA |
| TdT buffer | BOEHRINGER MANNHEIM |
| Trisodium citrate | SIGMA |
| dUTP/fluorescein | PROMEGA |
| Becton Dickinson FACStar, Becton Dickinson FACScan, LYSIS II & cellfit software | Becton Dickinson UK Ltd, |

Animal experiments

HsdOla:ICRF-nu mice

standard diet

slow-release oestrogen pellet

(0.72 mg, released over 60 days)

slow-release tamoxifen pellet

(2.5 mg released over 60 days)

hypnorm

hypnovel

MICHEL clips

5ml Plastipak syringe

23G Yale Microlance needle

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GIBCO Life Technologies Limited, 3 Fountain Drive, Inchinnan Business Park, PAISLEY, UK

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Innovative Research of America, Ohio, U.S.A.

Jensen Animal Health, Grove, Oxon, U.K.

NBS Biologicals, New Brunswick Scientific (UK) Limited, Edison House, 163 Dixons Hill Road,
North Mymms, HATFIELD, UK.

Novocastra Laboratories Limited, 24 Claremont Place, NEWCASTLE-UPON-TYNE, UK.

Roche Products Ltd., P.O. Box 8, WELWYN GARDEN CITY, HERTS, U.K.

Sigma Diagnostics, St. Louis, USA.

Methods

Histology

A portion of each tumour was fixed for at least 24 hours in paraformaldehyde and then routinely processed into paraffin blocks. The blocks were numbered as follows ; all the ZR-75-1 tumours from study 1 were randomly allocated a sequential number from 1. The MDA-MB-231 tumours from passages 2 & 7 were randomly allocated a sequential number from 200. Finally the ZR-75-1 and MDA-MB-231 tumours from the combined experiment (passages 36 & 9 respectively) were randomly allocated a number from 100. 3 μ m sections were cut and mounted onto glass slides, and routine Haematoxylin and Eosin staining was performed. For those tumours with sufficient tissue, the remainder of the sample was kept in liquid N₂ for subsequent use, including the cutting of 5 μ m thick frozen sections on a cryostat, which were then stored at -80°C.

The routine H & E staining afforded the opportunity to confirm that the sections contained tumour, which was important for the xenografts. All tumours from which data are presented contained tumour - however the two control ZR-75-1 tumours from passage 28 which had very poor growth, and were therefore excluded, did in fact contain only scattered tumour cells, and therefore their exclusion also appears justified on histological grounds.

Apoptosis & Mitosis

Given the difficulties of defining an optimum method for apoptotic assessment, it was felt appropriate to rely only on the classical morphological measures. These were to be assessed by one observer (DAC), after instruction by an experienced pathologist, Dr. T.J.Anderson. A proportion of the sections assessed for apoptosis from both the clinical cases and the mouse xenografts were counted by both observers in order to provide a measure of reproducibility for the method.

The microscopy of the xenograft sections was performed on sections stained with routine Haematoxylin and Eosin. The observer was blinded to the treatment status of the animal, and to the day on which it was removed. It was not possible to be blinded to the cell line used, as ZR-75-1 and MDA-MB-231 tumours were morphologically distinct, with the former having more obvious patterns of differentiation including islands of tumour surrounded by stromal/vascular channels. In order to assess the apoptotic (and mitotic) indices, 10 random high-power (x400) fields were examined using the HOME microscope (Brugal et al(1992)) by one observer (DAC). This computer-linked microscope records which cells are marked, permitting later confirmation by the second observer

(TJA). Any field that abutted onto or contained an area of necrosis was not used, because on the peripheries there were sometimes cells whose nuclear morphology suggested apoptosis but without the classic contracted eosinophilic cytoplasm. In order to avoid confusion and possible bias it was felt appropriate to ignore these fields (as in (Arends et al(1994))). For some tumours the section was too small to identify 10 distinct fields, and in these cases all the tumour cells in one section were examined. For the second experiment, which involved animals bearing both ZR-75-1 and MDA-MB-231 tumours, most tumours were smaller than in the previous experiment, and thus only 8 fields were used to standardise the number of fields examined between different tumour types removed at different times.

Apoptotic cells were identified by their morphometric appearance (Cohen, (1993)) (see figure 4), and mitotic figures were defined as by Baak (Jannink et al(1996)), using the convention that two parallel chromosome clots are counted as separate mitoses (Jannink et al(1996)) (see figure 5). The total number of cells in the 10 high power fields was estimated by counting the number within a representative region of each field of view (usually about one third), whose area (A) was then measured by the HOME microscope (Brugal et al(1992)). Multiplying the number (N) counted in this region by the ratio of the total area of the section (A_0) to the area of the smaller region gave an approximation to the total number of cells for that field ($= A \times A_0 / N$); the whole section was counted when the tumour was too small to be divided into 10 fields. The apoptotic and mitotic indices (AI and MI respectively) were defined as the ratio of the number of apoptotic cells (or mitotic figures) to the total number of tumour cells. The apoptotic:mitotic ratio was the ratio of the total counts for each section (with the exception of those tumours where no mitoses were seen in the 10 random high power fields, when the ratio was underestimated as being equal to the apoptotic count for the 10 fields; this situation only arose in a single, regressing, control ZR-75-1 tumour, which had been removed on day 28, and in 34/61 treated ZR-75-1 tumours, removed on days 7 - 28).

For the clinical breast cancers, the HOME microscope was used, but in order to permit a comparison between tissue taken from different tumours both before and after treatment, a minimum of 5 high-power fields was examined, until at least 1 000 malignant cells had been viewed; even so there were five post-treatment tumours for which there was insufficient tissue available to examine more than 1 000 malignant cells. The same criteria for apoptotic and necrotic cells were employed as for the xenografts. The apoptotic : mitotic ratio was also defined as for the xenografts, and in the absence of any mitotic figures being noted in the fields examined, the ratio was underestimated to be equal to the number of apoptotic figures counted.

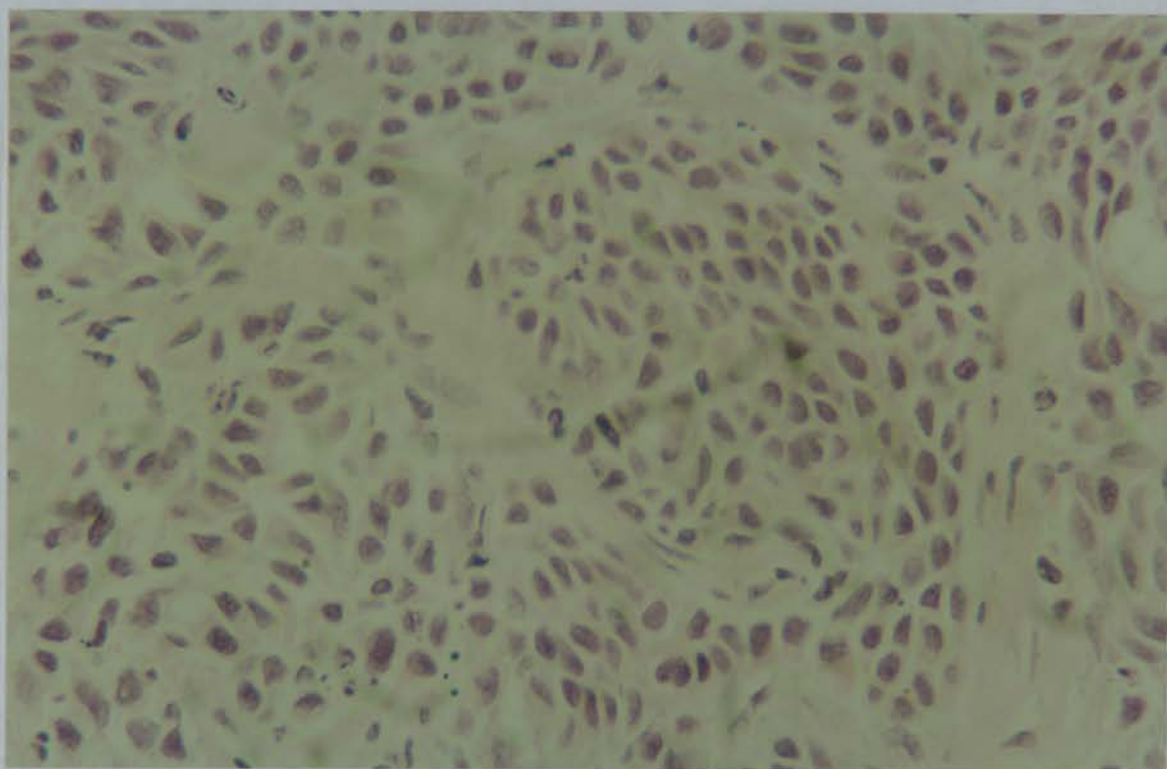


Figure 4 Photomicrograph of a ZR-75-1 xenograft with an apoptotic cell seen at the centre of the plate, with a horseshoe-shaped nucleus. Further typical apoptotic cells are to be seen superiorly and towards the bottom and top left corners (H & E staining, x 400 magnification)

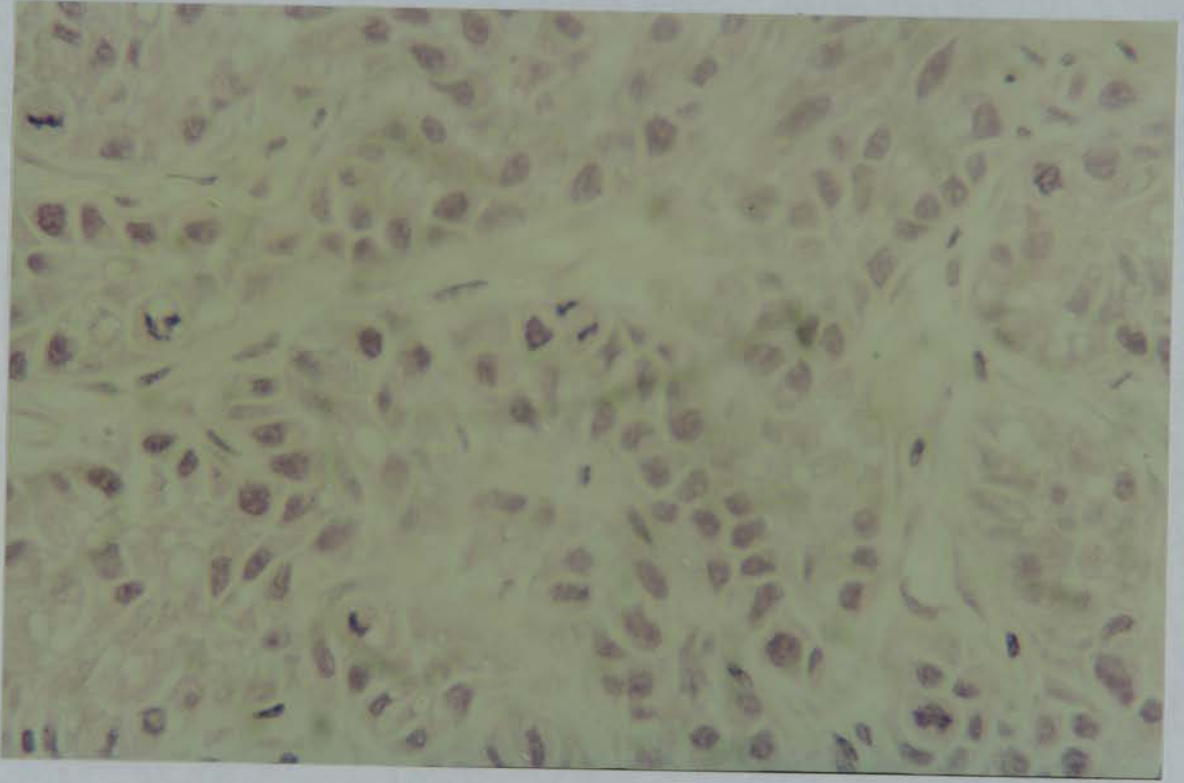


Figure 5 Photomicrograph of a clinical breast cancer with a mitotic cell in telophase seen at the centre of the plate. Further typical mitotic figures are to be seen in the top left and bottom right corners (H & E staining, x 400 magnification)

Necrosis

ZR-75-1 tumours from the first experiment (study 1) were assessed to determine the proportion of each tumour that was necrotic. Necrotic tissue was identified under light microscopy by the presence of cells that were morphologically necrotic (Borgers et al(1987); Trump et al(1982); Trump et al(1984); Wyllie, (1981)), with or without adjacent hemorrhage (see figure 6). The HOME software was employed to calculate the total area of necrosis and the total area of tumour in the section (excluding areas of pure stroma). The proportion of each tumour that was necrotic was defined as the ratio of the area of necrotic tissue to the total area of the tumour within the section.

A similar study could not be consistently conducted on the MDA-MB-231 tumours. This was because they tended to have large central necrotic cores, and on bisection and subsequent fixation of the tumour, the volume (and thus cross-sectional area) of the necrotic core was variably reduced.

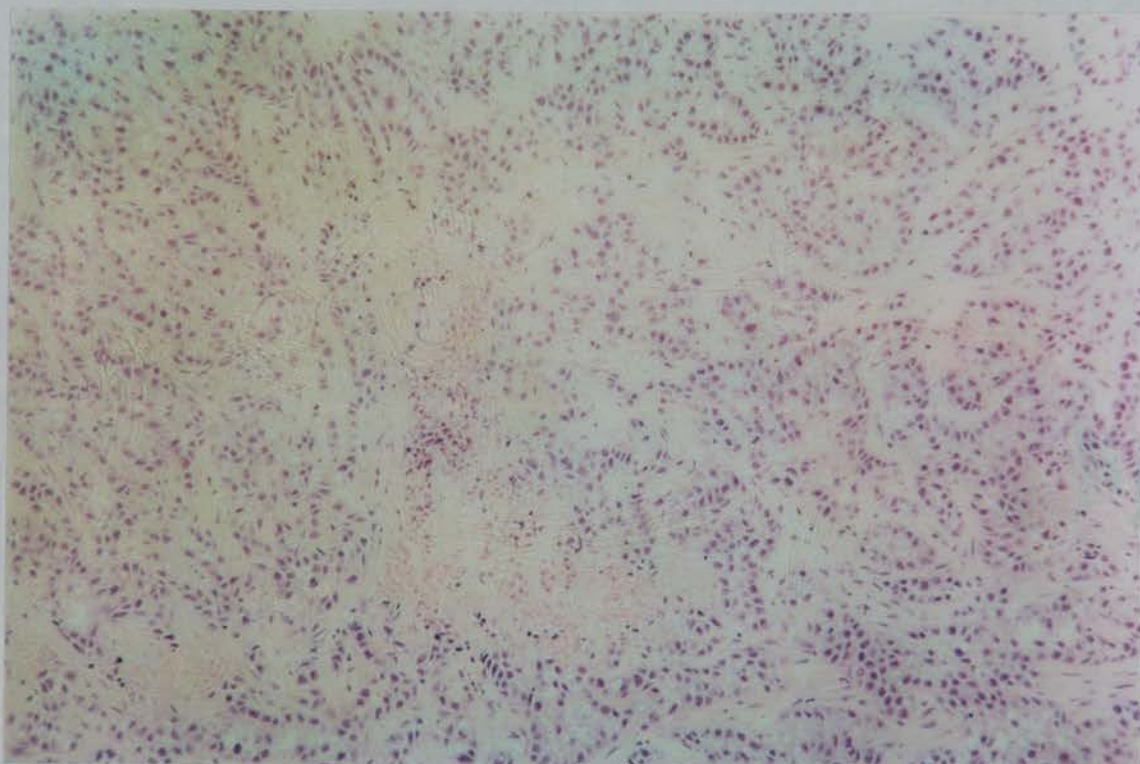


Figure 6 Photomicrograph of a ZR-75-1 xenograft, with an “L”-shaped region of necrosis visible in the centre of the plate. A further, smaller region is seen towards the bottom left corner. (H & E staining, x 100 magnification)

Immunohistochemistry

Paraffin-embedded sections

Semi-quantitative detection of antigen expression in tissue is best performed using immunohistochemistry. Its main advantage over the more quantitative methods (such as Western blotting) is that the site of expression can be visualised, and thus an observer can restrict the assessment to subgroups of cells - which in most cases is to malignant cells. The methodology used for immunohistochemical staining with antibodies is based on the original description (Sternberger, (1974)), but with some later refinements (Cattoretti et al(1988a); Hsu et al(1981); Shi et al(1991)). All staining runs included a section known to be positive (usually a routine formalin-fixed breast tumour, except for the bcl-2 runs, when a section of normal tonsil was employed), as well as a section known to be negative. In each run there were 20 - 24 sections, which always included sections from all time-points, and both treated and untreated tumours. Furthermore all slides had at least two sections, so that one could be used as a negative control by simply omitting the primary antibody. Any run in which the positive control did not stain or the negative control did stain was rejected.

For paraffin-embedded material, the tissue was first de-waxed, by sequentially placing the sections (usually mounted on lysine-coated slides) in xylene, pure alcohol, 95% alcohol, 70% alcohol and water, each for about 5 minutes. Endogenous peroxidase activity was blocked by a 15 minute incubation at room temperature with 30% Hydrogen peroxide. For some antigens (see page 54), a retrieval technique was necessary, performed by microwaving (Shi et al(1991)). The sections were immersed in 500 ml of 0.01M Citric Acid, pH 6.0 (achieved by addition of 1 M NaOH) and microwaved for 3 periods of 5 minutes each in a standard 750 W domestic microwave with turntable (additional hot distilled water was added after each 5 minute period if the sections had become uncovered). The sections were subsequently left to stand in the hot citric acid for a further 20 minutes, and transferred to TBS for 5 minutes. Non-specific antibody binding was reduced by incubation for 20 minutes in 20 % foetal calf serum diluted in TBS (20% FCS). Primary antibody solution (50 - 100 μ l) (see page 54 for dilutions) was placed over the sections, with the negative control sections being left covered by 20% FCS, and incubated either at room temperature for 1 hour or overnight at 4°C (see page 54). The sections were then washed x 2 with TBS for 5 minutes, followed by an hour's incubation with a secondary, biotinylated rabbit anti-mouse, antibody. After further washing x 2 with TBS, the sections were incubated for 20 minutes in ABC complex (3 μ l of avidin and 3 μ l of biotinylated peroxidase in 1ml of TBS (Cattoretti et al(1988a))), rinsed in TBS, and covered with 100 μ l of DAB (1mg/ml) containing 1 % Hydrogen Peroxide for 2 - 5 minutes (the actual length of time was determined by the rate of background staining appearing in the control sections). Normally sections from xenografts required less exposure than clinical specimens because

they were more prone to non-specific background staining by DAB, particularly after microwaving. The sections were then immediately rinsed in running water, counterstained with Haematoxylin for 20 seconds, dehydrated, cleared and mounted with DPX mountant before covering with a cover slip for viewing.

The sections were then "scored" by viewing 10 random high-power fields under a ZEISS microscope, excluding necrotic regions. The total number of malignant epithelial cells within each field was counted, as well as the number of cells that were clearly stained brown with the DAB ; and the ratio of these two counts calculated. Examples of stained sections are shown in figures 7 and 8. Figure 7 illustrates the results of immunohistochemistry for bcl-2 in a clinical breast cancer, with in this particular example virtually all the tumour cells are positive, and this tumour would have been given a bcl-2 score of 3. In contrast, figure 8 demonstrates the pattern typically seen with MIB-1 expression in the xenografts, where the staining was not homogenous across the section. Regions in which positive cells were to be found more frequently were often, but not exclusively, located towards the periphery of the tumour as is the case for the tumour shown.

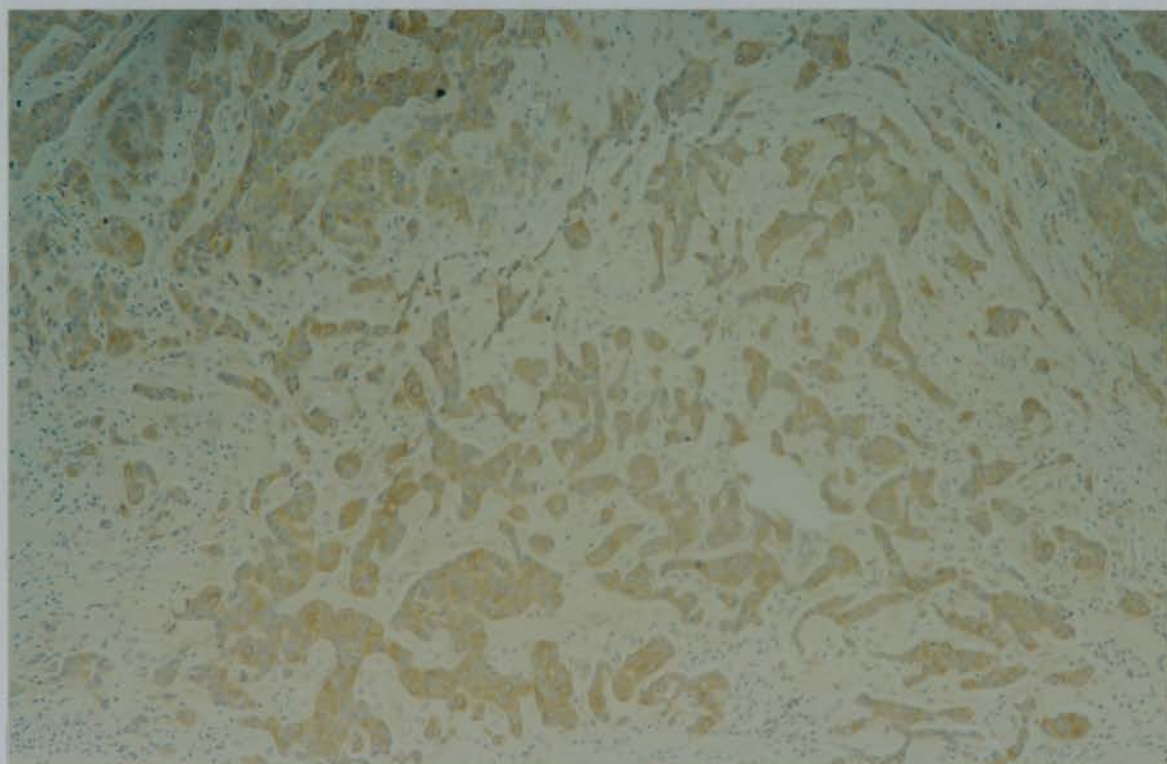


Figure 7 Photomicrograph of a clinical breast cancer stained for bcl-2 expression to which a bcl-2 score of 3 was given (x 100 magnification).

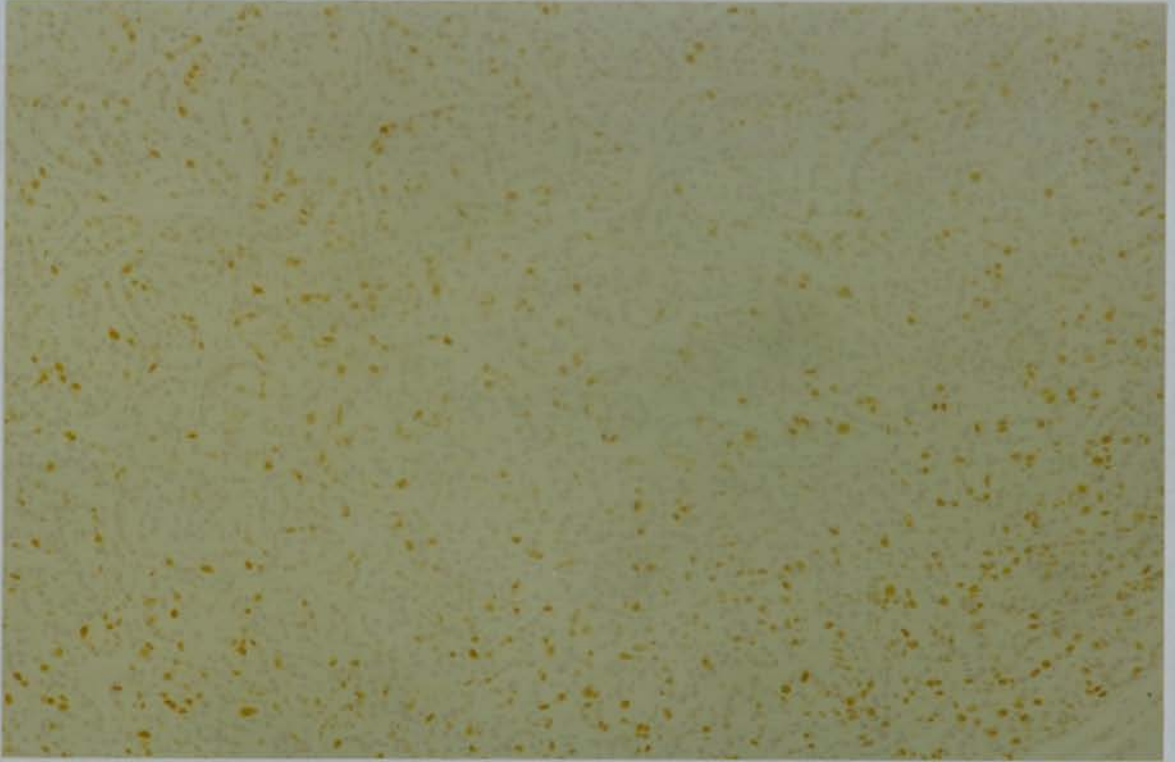


Figure 8 Photomicrograph of a ZR-75-1 xenograft stained for MIB-1 expression, illustrating the variation in staining across the section, maximal at the periphery (the surrounding mouse stromal tissue can just be seen at the bottom right of the section) (x 100 magnification).

Vasculature

Assessment of the xenograft microvasculature was performed on 5 μ m thick frozen sections, with a directly fluorescein-conjugated secondary antibody. (This was despite attempts to use paraffin-embedded sections and antigen retrieval by microwaving or pre-treatment with trypsin or proteases). Thus there was no requirement for rehydration (or dehydration), blocking of peroxidase or antigen retrieval techniques. The sections were therefore brought up to room temperature from the -80°C freezer in which they had been stored, incubated with 20% FCS/ 80% PBS for 20 minutes, covered with the MEC 13.3 antibody for 30 minutes (diluted 1 in 2 with 20% FCS), washed in 2 rinses of PBS, and then incubated with the secondary antibody, fluorescein-conjugated goat anti-rat. A counter-stain for the nuclei was provided by a further 5 minute incubation in 50 ml PBS containing 100 μ l of propidium iodide. The sections were then viewed under a ZEISS fluorescent microscope ; an example of a ZR-75-1 xenograft section is shown in figure 9.

The methodology employed for assessment of the micro-vascular density was to measure the proportion of the area of each section that expressed the vascular antigen, a murine equivalent of CD-31 (Vecchi et al (1994)). Therefore a representative region of each section corresponding approximately to a single low-power (x 10) field was viewed. The total area of the field of view was calculated using the H.O.M.E. software, not including areas that did not containing tumour or were frankly necrotic (as assessed under 584 - 607 nm which permitted visualisation of the nuclei by fluorescence of the PI). Then the marked region was viewed under high power (x 40), at 515 - 545 nm (permitting the fluorescein to be visible), and all areas of vasculature marked, and their total area calculated. Defining areas of pure vasculature sometimes required the frequency to be changed (by changing filters) to ensure that the area did not include tumour tissue. Thus the area of vasculature was measured, not the area of tumour encompassed by vessels. The vascular density was the ratio of the positive area to the total area viewed.

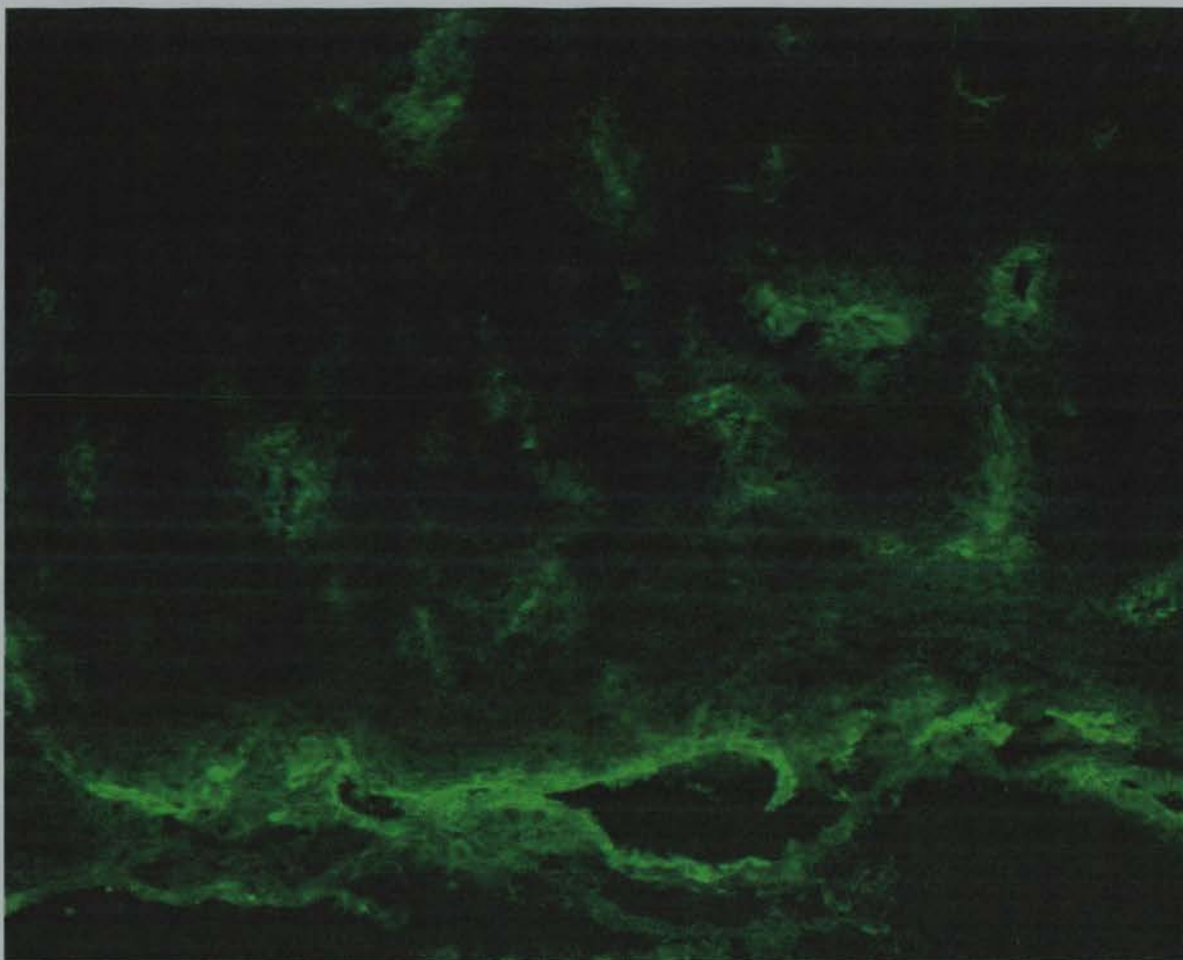


Figure 9 Digitally captured image of fluorescein-stained vaculature in a ZR-75-1 xenograft. The vessels in stroma surrounding the tumour are to be seen towards the bottom of the section. One particularly clear intratumoral vessel can be seen in the upper right corner. (X 200 magnification)

Flow-cytometry

DNA cell cycle analysis of FNAs

For DNA content analysis, the method employed was originally described by Vindeløv (Vindeløv et al(1982)). Five standard solutions were required :

Citrate Buffer 85.5g sucrose and 11.76g trisodium citrate are dissolved in 800ml distilled H₂O; 50 ml DMSO are added, the pH adjusted to 7.6 and distilled H₂O added up to a volume of 1litre.

Stock solution 2 g trisodium citrate, 121 mg Tris, 1044 g spermine tetrachloride and 2 ml Nonidet P40 are dissolved in distilled water to 2 litre, with the pH adjusted to 7.6.

Working solutions (all can be stored frozen and brought to room temperature before use) :

Solution A 15 mg trypsin in 500ml stock solution, pH adjusted to 7.6.

Solution B 250 mg trypsin inhibitor and 50 mg RNAase A in 500 ml stock solution.

Solution C 208 mg propidium iodide and 500 mg spermine tetrachloride in 500 ml stock solution.

After thawing, the FNA was centrifuged at 500 g for 5 minutes; washed in PBS, and resuspended in 100 µl citrate buffer. Solution A (225 µl) was then added to the buffer/cell mixture (100 µl), and the mixture gently inverted and incubated at room temperature for 10 minutes. Solution B (170 µl) was added, gently mixed and incubated at room temperature for 10 minutes. Finally solution C (175 µl) is added, the mixture inverted and incubated on ice for 10 minutes. Analysis of the preparation was performed on a B.D. FACScan, using the red (584 - 607 nm) channel. The region to be counted was gated on area versus width in order to eliminate cell doublets that could otherwise be inadvertently included in the G₂/M population. Figure 10 illustrates this for a ZR-75-1 xenograft.

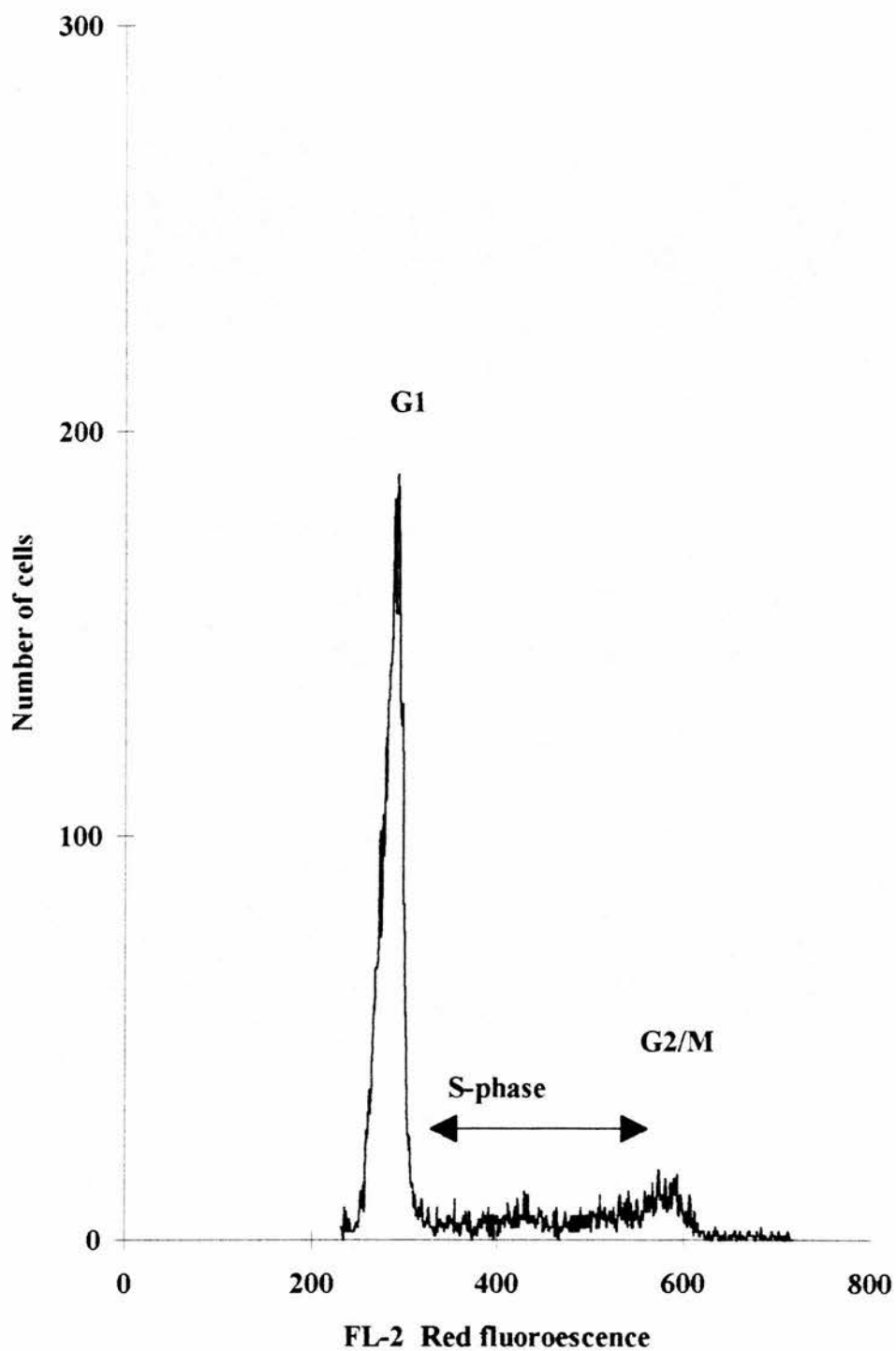


Figure 10 DNA histogram from an FNA taken from an untreated ZR-75-1 xenograft, with DNA content measured as red fluorescence

Cell-death assay (FACS-TUNEL)

After thawing, an aliquot of the FNA was suspended in 2 ml of 1% paraformaldehyde for 10 minutes at room temperature. This mixture was then centrifuged at 500g for 4 minutes, the supernatant discarded and the pellet re-suspended in 70% ethanol at room temperature. After a further 10 minutes this was re-centrifuged, washed x2 in TBS, re-centrifuged, and the cell pellet re-suspended in 40 µl of 20% 5xTdT buffer diluted in distilled water and allowed to stand for 10 minutes at room temperature. The suspension was centrifuged for 4 minutes at 500g, and the pellet incubated for 2 hours at 37°C in 25 µl reaction mixture (100 µl of which contains 20 µl 5xTdT buffer, 78 µl distilled water, 1 µl dUTP/fluorescein and 1 µl TdT (=15 units)). Finally the cells were washed twice in TBS, re-suspended in 1ml TBS and left to stand for 15 minutes at room temperature.

Flow cytometric analysis was performed on a B.D. FACScan, exciting at 488nm using an argon laser. Forward and Side scatter, and green fluorescence (515 - 545 nm) were collected on a log scale, and analysed using LYSYS-II software running on a HP-3000 computer. Populations were identified on a scatter-plot of green fluorescence against side scatter (see figure 11). The apoptotic population was taken as the identifiable group of cells with high green fluorescence and intermediate side scatter. Necrotic cells, which were not counted as apoptotic, were identified as having low side scatter and intermediate green fluorescence. DNA histograms were performed on the samples that had been stained with PI, and analysed using the "cellfit" software, under red fluorescence (584 - 608 - nm).

A further refinement of this method, applied to some of the FNAs with sufficient residual material, was to try and remove the necrotic and late-stage apoptotic cells by pre-treatment with enzymes that can be excluded by viable and early-stage apoptotic cells. This is achieved, after thawing, but before exposure to paraformaldehyde, by incubating the cells with 0.25% trypsin and 100 µg/ml DNAase I in RPMI for 30 minutes at room temperature as described previously (Darzynkiewicz et al(1984)).

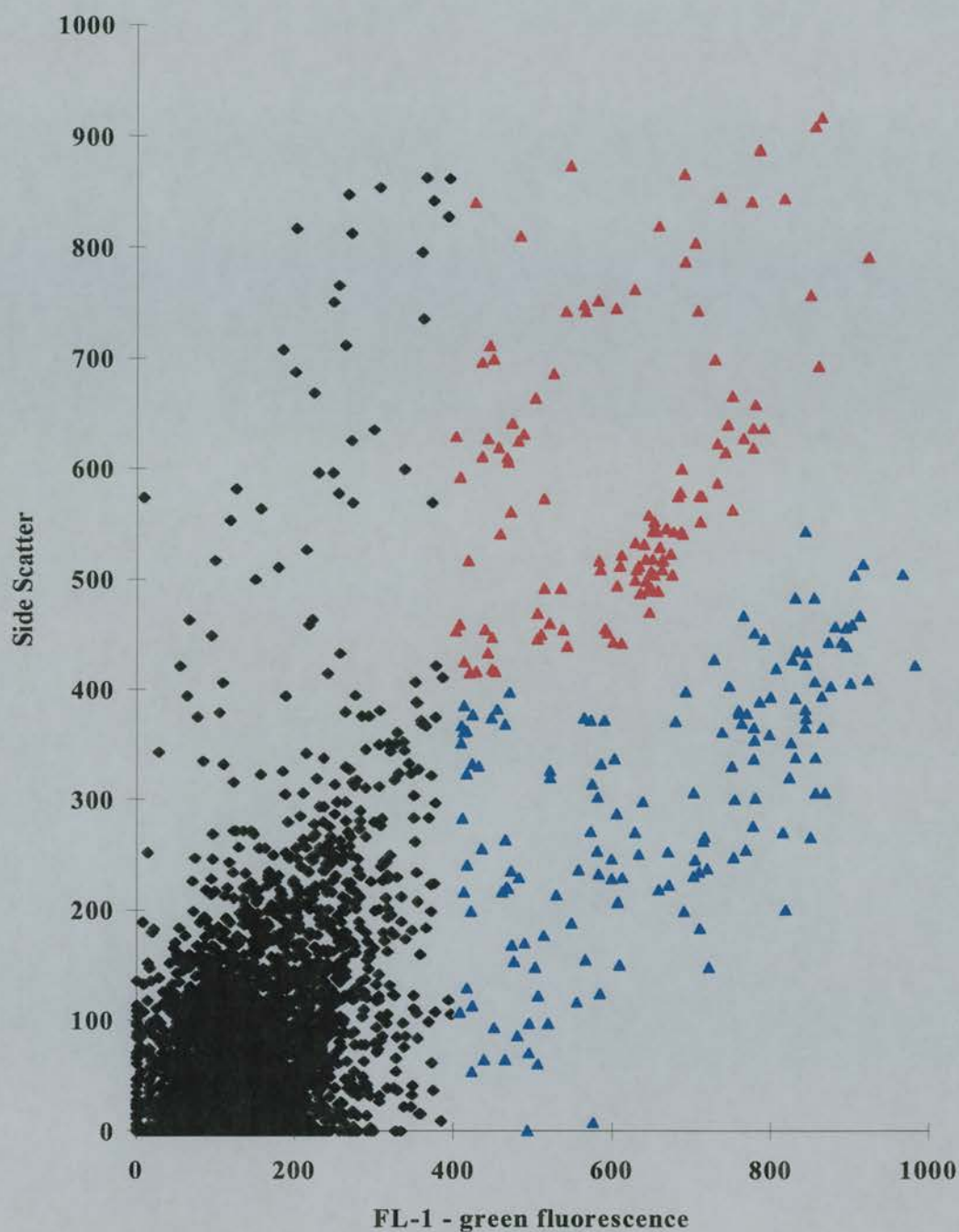


Figure 11 Fluoro-TUNEL assay of a FNA from a ZR-75-1 xenograft. Apoptotic cells are shown in red, having high DNA-fragmentation (high FL-1 fluorescence) and high side-scatter. Cells with low side-scatter and high DNA-fragmentation are shown in blue, and represent a mixture of late apoptotic cells, necrotic cells and individual apoptotic bodies. As such they are not included in the overall percentage of apoptotic cells.

In order to confirm that cells identified as apoptotic by this technique would have at least included those similarly classified as apoptotic by morphological criteria, a fluorescent microscope (ZEISS, Germany) was used. Firstly after staining by the above procedure, cells from both MDA-MB-231 and ZR-75-1 FNAs were analysed on a BD FACStar cell sorter. The populations considered to be apoptotic and necrotic by the above criteria were gated out. They were then smeared onto a lysine-coated slide, and examined for green fluorescence using a fluorescent microscope. Apoptotic cells (as defined by their chromatin morphology) were seen in the high green, intermediate side scatter group, but not every cell showed clear chromatin condensation : which is not unexpected since DNA fragmentation occurs before chromatin condensation (Schwartzman et al(1993)). In contrast no morphologically apoptotic cells were seen in the low side scatter, intermediate green fluorescence group of cells. For a further test, several sections of ZR-75-1 tumours mounted on lysine-coated slides, were prepared with the same reagents. The sections were then examined under the same fluorescent microscope. Again, nuclei that were clearly apoptotic were identified, although there were also confluent areas of fluorescent cells that were probably necrotic, together with scattered cells that did not have blebbed chromatin that could have represented cells at an earlier stage of apoptosis.

Growth modelling

This was performed using a suite of programmes written in FORTRAN 77, called ADAPT. The user specifies the function (equation III on page 43), as well as the data points, which consist of the time and log (volume). All estimations were run from the same starting values of $\alpha = 0.5$, $\beta = 0.05$ and $\tau = 10$. No formal tests of stability were conducted, as the Gompertz function is continuous, as are all its derivatives, but informal tests showed that the modelling was robust with respect to the actual starting values.

A minimum of three recorded untreated volumes are required to fit a Gompertz growth curve, and this was not always the case, particularly for the tumours from the second study (as due to unforeseen circumstances, tumour volumes were only measured three and one weeks before starting therapy, as well as on the day treatment was begun). Therefore certain tumours could not have individual growth curves fitted, and as discussed on page 173, for some experiments averaged growth curves were used. However, since the volumes were log-normally distributed, and the function in equation III is based on the log of the volumes, it was the average of the log of the volumes that was used. If the tumour was too small on day -21 for an accurate measurement, omitting this value from the average would increase the value on day -21, which would then underestimate the curvature of the average growth curve, possibly thus overestimating the subsequent growth. In order to avoid this, the smallest possible volume measurement (0.002 cm^3 , corresponding to a diameter of just under 0.15 cm, the smallest diameter recorded for any tumour) was given to tumours which had been too small to be measured, but in which a measurement was available at the next time point, confirming that tumour was present.

Data and statistical analysis

Tests for normality of data were conducted (combining data from different passages) for each parameter in turn (e.g. apoptotic index, mitotic index etc.). The following variables were found to be normally distributed :

log_e(apoptotic:mitotic ratio)
log_e(ER)
log_e(% apoptotic cells by FACS)
log_e(xenograft volumes)
% vasculature
% cells in S-phase by FACS
% cells in G₁ by FACS
% cells in G₂/M by FACS

For these variables therefore parametric tests could be employed, and correlations performed using the Pearson's correlation coefficient. For the remaining variables, non-parametric tests (usually Mann-Whitney or non-parametric trend analyses) and the Spearman's rank correlation were used.

student-t tests and non-parametric trend analyses (after (Cuzick, (1985)))

Excel for Windows version 5.0

Microsoft Corporation, Redmond, WA, U.S.A.

Mann-Whitney, Spearman & Pearson rank & Normality tests

Minitab release 5.1.1

Minitab Inc., State College, PA, U.S.A.

Growth curve predictions

ADAPT II

Biomedical Simulations Resource,
University of Southern California, CA, U.S.A.

Results

- 1 Methodology
 - 1.1 cell counting
 - 1.2 apoptosis and mitosis
 - 1.2.1 clinical
 - 1.2.2 xenograft
 - 1.3 Discussion

- 2 Clinical Tumours
 - 2.1 Response
 - 2.2 Oestrogen Receptor concentration
 - 2.3 Bcl-2 expression
 - 2.4 Apoptosis
 - 2.5 Mitosis
 - 2.6 Apoptotic:Mitotic ratio
 - 2.7 Discussion

- 3 ZR-75-1 xenografts
 - 3.1 Growth
 - 3.2 Apoptosis & Mitosis
 - 3.3 MIB-1,bcl-2, p53 & c-erb-B2 expression
 - 3.4 Necrosis
 - 3.5 Vascular density
 - 3.6 Fine Needle Aspirates
 - 3.6.1 Apoptosis
 - 3.6.2 Cell-cycle analysis
 - 3.7 Correlations
 - 3.8 Discussion

- 4 MDA-MB-231 xenografts
 - 4.1 Growth
 - 4.2 Apoptosis (& Necrosis)
 - 4.3 Mitosis
 - 4.4 Apoptotic/Mitotic ratio
 - 4.5 Vascular density
 - 4.6 FACS data
 - 4.7 Correlations
 - 4.8 Discussion

- 5 Synchronously grown ZR-75-1 and MDA-MB-231 xenografts
 - 5.1 Growth data
 - 5.2 Apoptosis, mitosis and the Apoptotic:Mitotic ratio
 - 5.3 Discussion

- 6 Sequential Fine Needle Aspirates of ZR-75-1 and MDA-MB-231 xenografts
 - 6.1 Growth
 - 6.2 FACS data
 - 6.2.1 S-phase & G2/M
 - 6.2.2 Apoptosis & Apoptosis:G2/M ratio
 - 6.3 Discussion

- 7 Analysis of individual ZR-75-1 and MDA-MB-231 xenografts
 - 7.1 Growth of ZR-75-1 xenografts
 - 7.2 Growth of MDA-MB-231 xenografts
 - 7.3 Correlations between individual tumour growth and biology
 - 7.4 Synchronously borne ZR-75-1 and MDA-MB-231 tumours
 - 7.5 ZR-75-1 and MDA-MB-231 tumours subjected to sequential FNAs
 - 7.6 Discussion

1. Methods

A major part of this study consists of histological examination of sections. The histological and immunohistochemical techniques employed are standard, and apart therefore from the routine inclusion of positive and negative controls require no further validations. However the assessment of the sections is subject to potential observer error, and confirmation therefore that the scoring of the sections is consistent and accurate is required. This section therefore details the assessments made to ensure the reproducibility of the methods employed for counting the total number of malignant cells examined, and the counting of the apoptotic cells in both xenografts and clinical tumours, and finally the mitotic figure counts for the clinical tumours.

1.1. Cell counting

In order to facilitate counting of the total number of cells in a section, a representative proportion of each high-power field was counted, and the total number of cells in that field estimated by multiplying the relative areas of the region counted and the total field. In order to ensure that this estimated total was accurate, the real total number of tumour cells in a random sample of 21 high-power fields was counted, including both treated and untreated tumours, as well as ZR-75-1 and MDA-MB-231 xenografts. Figure 1.1 shows the correlation, which is highly significant ($r_i = 0.821$, $p < 0.001$), and confirms that the use of an estimated total is sufficiently accurate. Similar relationships were found when restricting the comparison to either just ZR-75-1 xenografts ($r_i = 0.793$, $p < 0.01$) or MDA-MB-231 xenografts ($r_i = 0.799$, $p < 0.01$), and for treated ($r_i = 0.837$, $p < 0.01$) and untreated xenografts separately ($r_i = 0.783$, $p < 0.01$). The identification of a correlation does not necessarily confirm the visual impression that the two methods of counting are equivalent, and this can be determined by calculation of the closeness of the two methods of scoring, known as the repeatability. For all sections, this is equal to 85 cells, and there is only one tumour where the difference in absolute values between the two methods exceeds this value. When considering the subgroups, there is one treated tumour, one untreated tumour, and one MDA-MB-231 tumour, but no ZR-75-1 tumour whose difference between the two methods of counting exceeds the respective repeatability. Thus overall there is very good correlation between the two methods.

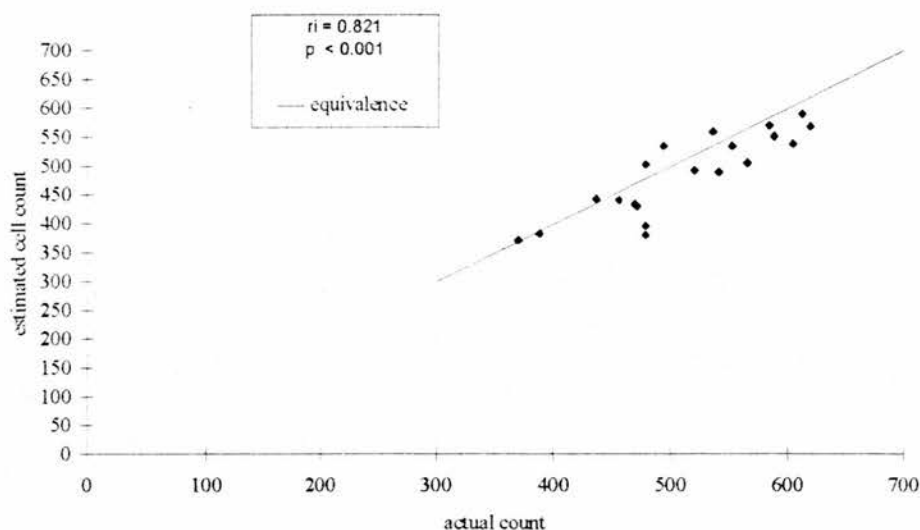


Figure 1.1 Comparison of estimated and actual high-power field cell counts for xenografts

1.2 Apoptosis and Mitosis

Any scoring system that is based on cellular appearance will be necessarily in part subjective. For immunohistochemistry, the criterion of positivity is simply the recognition that a cell or (in the case of MIB-1) its nucleus is a different colour from the others. However for the identification of apoptotic and mitotic figures, which were determined by their morphology, there is the possibility that the criteria employed by one observer may be different from those of another observer. Therefore a number of sections were re-examined, both by myself and also by an experienced pathologist, Dr. T J Anderson, in order to confirm that the scoring was reproducible. The criteria for identifying mitotic figures is well documented and in routine clinical use, therefore comparison was only made for the clinical tumours to ensure equivalence of the two observers' scoring. However the morphological appearance of apoptotic cells is less widely applied, and does not form a part of any routine clinical assessment, and therefore the scores obtained in both clinical and xenograft tumours were compared. Similarly, both control and treated tumours were examined.

1.2.1 Clinical

Since every patient had two pathological specimens, the tumours examined for ratification of their apoptotic and mitotic indices were a selection of pairs ; for this comparison tumours were chosen such that they were representative of low, moderate and high apoptotic indices. The raw data are given in table 1. 1, and show an excellent correlation between the two scorers. It can be seen that two scores are identical in the majority of cases, and when dissimilar, either observer can count the higher number. The equivalence of the two scores is underlined by the fact that no mitotic score and only one apoptotic score exceeds the “repeatability”.

| | | Apoptoses | | Mitoses | |
|---------------|------|---------------|------------------|---------------|------------------|
| tumour | | DAC | TJA | DAC | TJA |
| BC49 | pre | 12 | 14 | 2 | 3 |
| BC169 | post | 11 | 11 | 1 | 1 |
| BJC79 | pre | 4 | 3 | 2 | 3 |
| BJC82 | post | 1 | 1 | 0 | 0 |
| BJC78 | pre | 3 | 3 | 1 | 1 |
| BJC86 | post | 2 | 2 | 0 | 0 |
| BJC48 | pre | 2 | 2 | 0 | 0 |
| BJC26 | post | 4 | 6 | 1 | 0 |
| BJC77 | pre | 7 | 6 | 4 | 4 |
| BJC84 | post | 2 | 2 | 1 | 1 |
| BJC74 | pre | 7 | 7 | 0 | 0 |
| 93/3196 | post | 14 | 13 | 6 | 6 |
| BJC45 | pre | 4 | 4 | 0 | 0 |
| BJC49 | post | 3 | 2 | 0 | 0 |
| BJC89 | pre | 9 | 5 | 3 | 2 |
| BJC93 | post | 10 | 10 | 0 | 0 |
| | | | no. exceeding | | no. exceeding |
| Repeatability | | 3 | 1 tumour | 1 | 0 tumour |
| Correlations | | $r_1 = 0.948$ | $p < 0.0001$ | $r_1 = 0.959$ | $p < 0.0001$ |

Table 1.1 Raw scores for counting apoptotic and mitotic figures in clinical breast cancers by two observers.

A further assessment of one observer's consistency (DAC) was performed by re-counting previously assessed sections, after an interval of some 6 months. Again, the data confirm that the criteria applied consistently, particularly for mitotic figures (see table 1.2.). Although on the majority of occasions the apoptotic scores are not identical for the two counts, there is no trend with time as the higher score can occur on either occasion. There is an excellent correlation for both apoptotic and mitotic scores, and in neither case does the difference between the two scores exceed the "repeatability".

| | Apoptosis | | Mitosis | |
|---------------|---------------|---------------|---------------|---------------|
| tumour | first count | second count | first count | second count |
| BC74 | 6 | 3 (1/2 area) | 0 | 0 |
| BJC49 | 3 | 2 | 0 | 0 |
| BJC56 | 6 | 4 | 2 | 1 |
| BJC64 | 8 | 8 | 0 | 0 |
| BJC80 | 3 | 2 | 0 | 0 |
| BJC81 | 20 | 22 | 2 | 2 |
| BJC83 | 4 | 3 | 0 | 0 |
| BJC84 | 2 | 3 | 1 | 1 |
| BJC85 | 2 | 1 | 0 | 0 |
| BJC89 | 9 | 7 | 3 | 3 |
| | | no. exceeding | | no. exceeding |
| Repeatability | 2.5 | 0 tumour | 1 | 0 tumour |
| Correlations | $r_i = 0.972$ | $p < 0.001$ | $r_i = 0.954$ | $p < 0.001$ |

Table 1.2 Raw scores for counting apoptotic and mitotic figures in clinical breast cancers by one observers on different occasions.

1.2.2. Xenografts

Sections from 13 ZR-75-1 tumours were double-counted for apoptotic cells. They were chosen at random, but ensuring that there were representatives of both treated and untreated tumours, as well as tumours from each time point. Five fields were counted for each tumour, except for two which were too small for 5 fields and were thus examined in toto and considered as one field. The data were analysed for all 57 individual fields separately, as well as for the thirteen tumours. Two tumours (DAC022 & DAC079) had little tumour material left, and thus instead of 5 fields, the whole section was examined. Table 1.3 gives the raw data, and confirms that there is excellent correlation between the apoptotic cell counts for the two observers. As with the clinical tumours, there is no consistent bias for one observer to count more cells than the other.

and the equivalence of the two counts is confirmed by the observation that in only one tumour (DAC028) does the difference between the two counts exceed the "repeatability".

| tumour | DAC | TJA |
|----------------------|---------------------------|----------------------------------|
| DAC001 | 38 | 36 |
| DAC019 | 23 | 23 |
| DAC020 | 13 | 15 |
| DAC022 | 0 | 0 |
| DAC028 | 43 | 37 |
| DAC033 | 12 | 13 |
| DAC036 | 48 | 49 |
| DAC047 | 16 | 12 |
| DAC050 | 21 | 21 |
| DAC053 | 21 | 21 |
| DAC066 | 29 | 27 |
| DAC079 | 0 | 0 |
| DAC083 | 16 | 17 |
| | for tumours | for all fields separately |
| Repeatability | 5 : 1 tumour exceeds | 2 : 1 exceeds |
| Correlation | $r_i = 0.985 ; p < 0.001$ | $r_i = 0.976 ; p < 0.0001$ |

Table 1.2 Raw scores for counting apoptotic figures in xenografted tumours by two observers.

1.3 Discussion

Histological methodologies were employed to determine the mitotic and apoptotic indices. They are subject to potential inter-observer variation because the observer is identifying cells as apoptotic or mitotic on the basis of differing morphologies. The criteria used to identify mitotic figures are those used in routine histology, and have been previously shown to be reproducible between a large number of different observers (van Diest et al(1992)). For the clinical tumours, inter and intra-observer variation was assessed, and for both comparisons a statistically significant correlation was found. Thus the reproducibility of the technique is confirmed. For the xenografts therefore, no further such studies were conducted.

The assessment of apoptosis is however more controversial, and has been less well studied. The criteria used to identify apoptotic cells are well described (Bellamy et al(1995)), but there has only been one study published to date that has assessed their reproducibility in breast cancer (Vandeschepop et al(1996)). This study elected to count

apoptotic cells in the most poorly differentiated regions of the tumour, in contrast to the present study in which fields were chosen at random within the tumour section. Furthermore, as necrosis is associated with higher grade tumours (Clayton, (1991)), there is the possibility that regions of necrosis might thus be included in the counts. Nevertheless, this study did report good intra- and inter-observer variability, but found that the highest power (x1000) gave the most consistent results. A comparative study in bladder carcinoma has been published (Lipponen et al(1994)), also with good intra- and inter-observer correlation. The issue of reproducibility was therefore considered in some detail in the present study. For the xenografts there was an excellent correlation between two observers, both for individual fields and when assessing the incidence throughout a tumour. Similarly for the clinical tumours, the intra- and inter-observer variation was low.

The two other measures that were based on histology were the measurement of necrosis and vasculature. The former simply required the area of necrotic tissue to be measured and compared with the area of the total section. The HOME software (Brugal et al(1992)) used to calculate the area is the result of a long collaboration between the industry and pathologists throughout the European Union (Dr. Gray, personal communication), and it was felt unnecessary to revalidate it. The identification of regions of necrosis was not considered to be potentially difficult. The measurement of the vasculature portion of the tumour is more subjective. However each section had an internal positive control, namely the vessels visible in the stroma at the periphery of the tumour, and they were always examined before studying the rest of the section. Furthermore there was another way to corroborate some of the vascular channels identified, since when examining the section under a different fluorescent filter so that the propidium iodide stained nuclei would be visible, it could then be seen that in many cases the apparent channels, seen as spaces between the PI stained tumour nuclei, coincided with the vessels identified by the fluorescent antibody. However no formal studies of the reproducibility of the measurements were conducted.

2. Clinical Tumours

This section reports the data obtained by histological (and in the case of ER, biochemical) evaluation of tumour material taken before and after three months' tamoxifen therapy in 50 elderly women with non-metastatic breast cancer, whose tumour response was assessed by four-weekly ultrasound measurements.

2.1 Response

Response was defined as the ratio of the original tumour volume to that measured after three months' therapy. The ultrasound rather than pathological volume was used in order to have a consistent definition over the duration of treatment. Table 2.1 confirms that the majority of the tumours responded to tamoxifen, with 19 (38%) of patients having more than a 50% volume regression, and in a further 29 (32%) there was definite regression but to less than 50% over the three months. Only 2 (4%) of tumours progressed and the remainder had stable disease.

| Non-responders | | Responders | |
|--|--------------------------------|--|---|
| progressed (> 10% volume increase) | stable ≤ 10% volume change) | minor response (10%< ≤ 50% volume decrease) | good response (> 50% volume decrease) |
| 2 (4%) | 13 (26%) | 16 (32%) | 19 (38%) |

Table 2.1 Pattern of response in 50 tumours after three months' tamoxifen therapy.

2.2 Oestrogen Receptor concentration

All tumours were ER positive (> 20 fmol/mg cytosolic protein), since that was an inclusion criterion for the study. The values of ER before and after three months' tamoxifen therapy are given in table 2.2 and figure 2.2.1, where it can be seen that there is a clear fall in ER value with therapy (p < 0.000001), with a higher value being seen after therapy in only two tumours (of whom one had a minor response and the other progressed).

| | minimum | median | mean | maximum |
|-----------------------|---------|--------|------|---------|
| pre-treatment (n=49) | 29 | 262 | 348 | 1496 |
| post-treatment (n=38) | 0 | 60 | 64 | 168 |

Table 2.2 Pre and post-treatment ER concentrations in 50 clinical ER positive breast cancers.

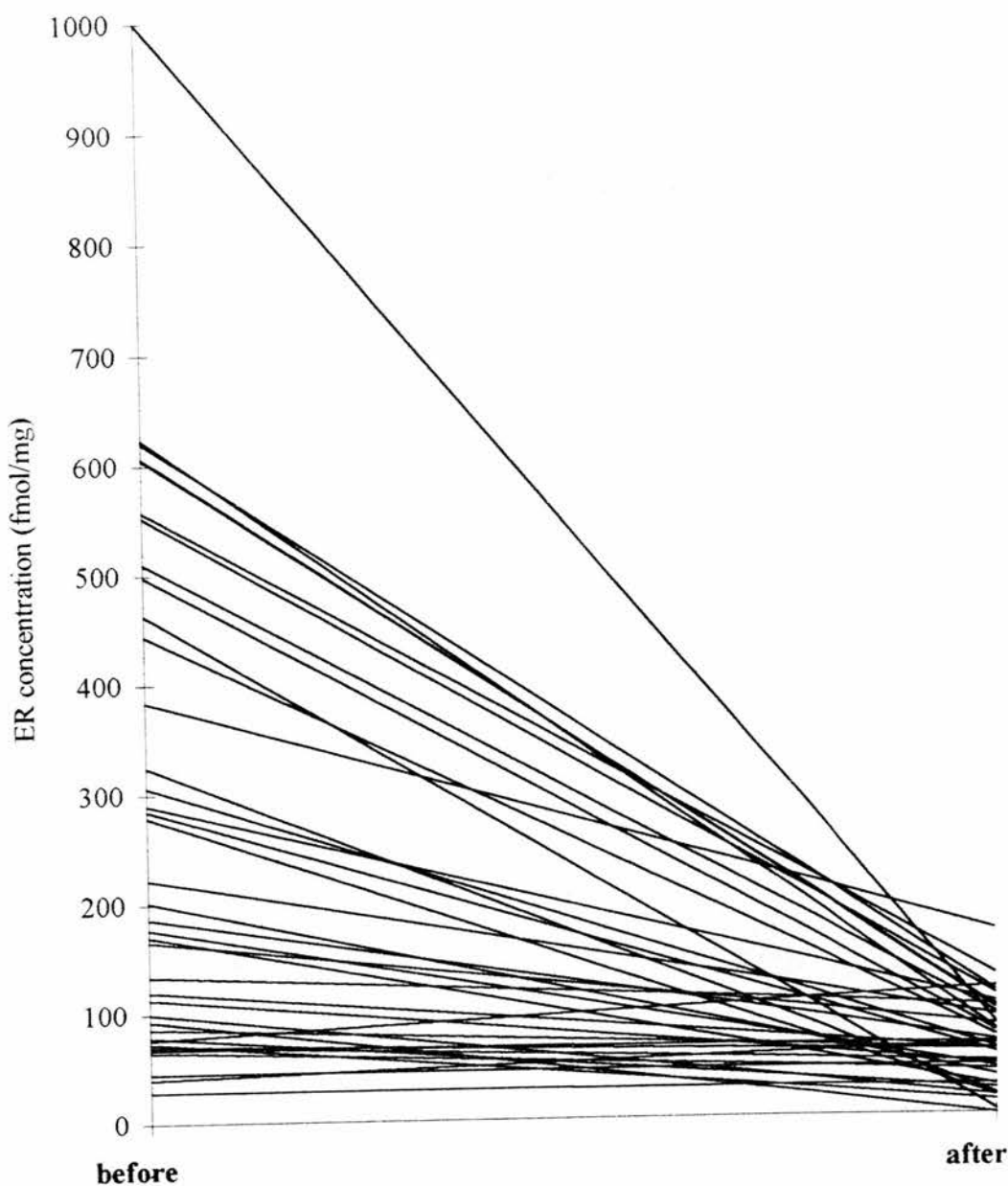


Figure 2.2.1 ER concentration before and after 3 months' tamoxifen therapy in clinical breast cancer

Figure 2.2.2 confirms that the pre-treatment, (although not the post-treatment) ER concentration is strongly correlated with response ($r = 0.595$, $p < 0.0001$). The change in ER also correlates with response as a continuous variable, as shown in figure 2.2.3, with the larger falls occurring in those tumours with a better response ($r = -0.516$, $p < 0.01$).

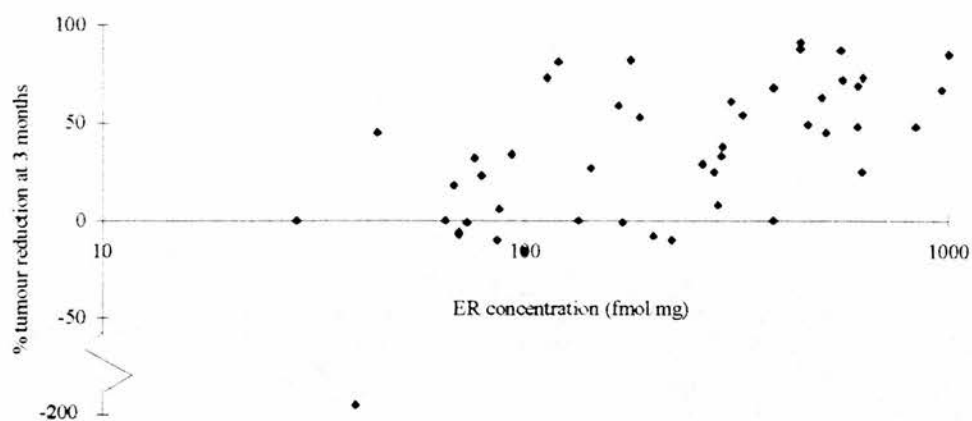


Figure 2.2.2 Pre-treatment ER and response in clinical breast cancers

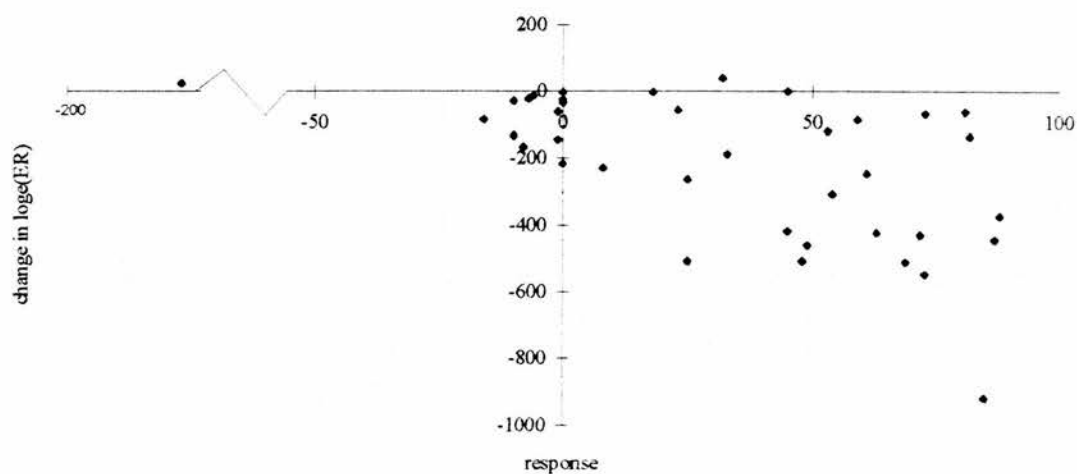


Figure 2.2.3. Change in ER concentration and tumour response after 3 months' tamoxifen therapy

2.3. Bcl-2 expression

Bcl-2 expression was scored on a semi-quantitative basis. The results for all the tumours are summarised in table 2.3, and confirm that the majority (88%) of tumours express bcl-2 before therapy, but the proportion falls to 74% after therapy, with the biggest reduction occurring in the proportion of tumours expressing bcl-2 in more than 75% of the malignant cells. The difference in expression after treatment is statistically significant ($p = 0.006$). Figure 2.3.1 illustrates the pattern of change in expression, from which it can be seen that the commonest pattern is a moderate reduction (a drop in score of 1).

| staining index | 0 < 5% cells | 1 5 - 25 % cells | 2 25 - 75 % cells | 3 > 75% cells |
|----------------|-----------------|---------------------|----------------------|------------------|
| pre-treatment | 6 (12%) | 18 (36%) | 12 (24%) | 14 (28%) |
| post-treatment | 13 (26%) | 17 (34%) | 11 (22%) | 8 (16%) |

Table 2.3 Bcl-2 expression in clinical tumours before and after 3 months' tamoxifen therapy.

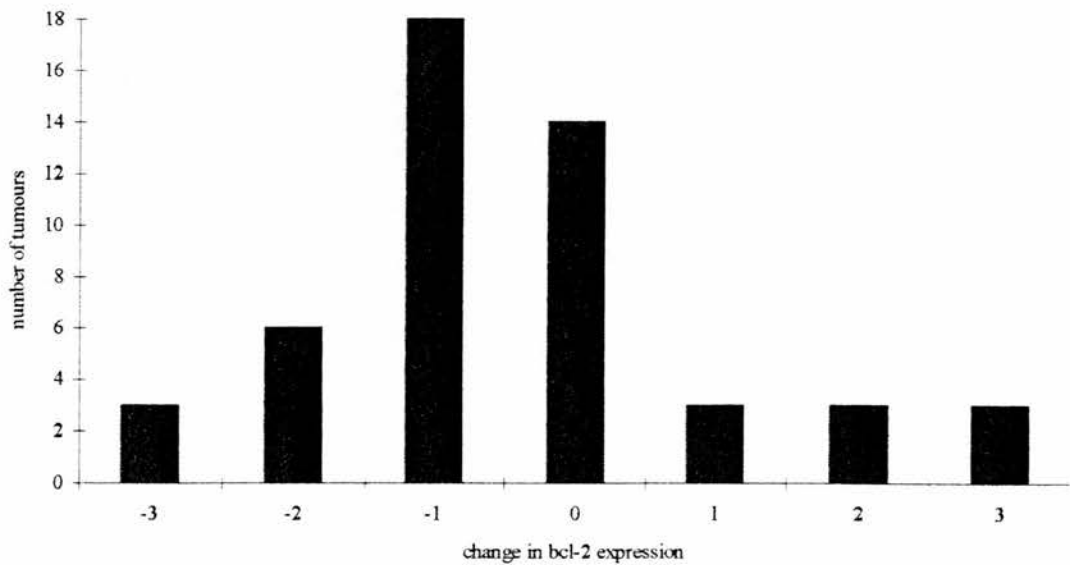


Figure 2.3.1. Change in bcl-2 expression with three months' tamoxifen therapy

There is a significant relation between initial bcl-2 expression and ER, with the tumours with the highest bcl-2 expression (i.e. a score of 3) having higher values of ER ($W = 465$, $p = 0.01$) (figure 2.3.2), as well as between initial bcl-2 expression and response, with higher expression occurring in tumours with a better response (see figure 2.3.3). ($z = 2.04$, $p < 0.05$). There are however no significant relationships between the post-treatment bcl-2 staining indices and either response or ER concentration.

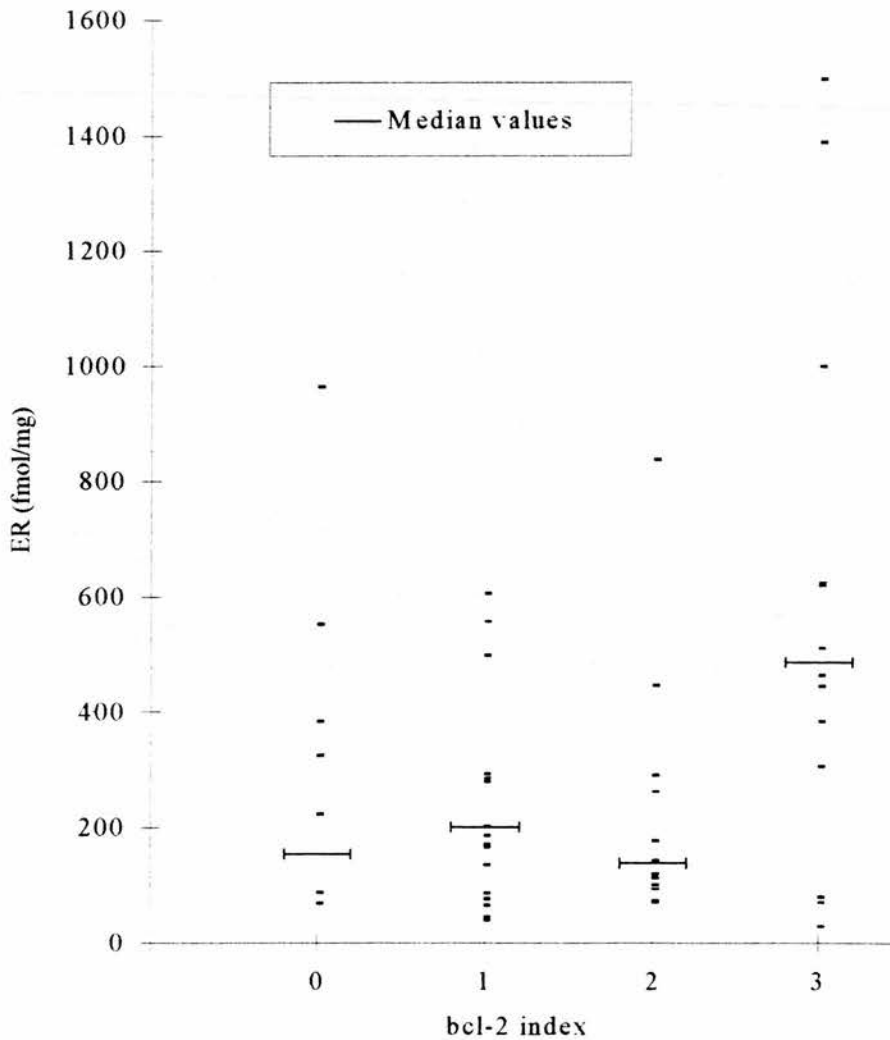


Figure 2.3.2 Pre-treatment bcl-2 expression and ER concentration in clinical breast cancers

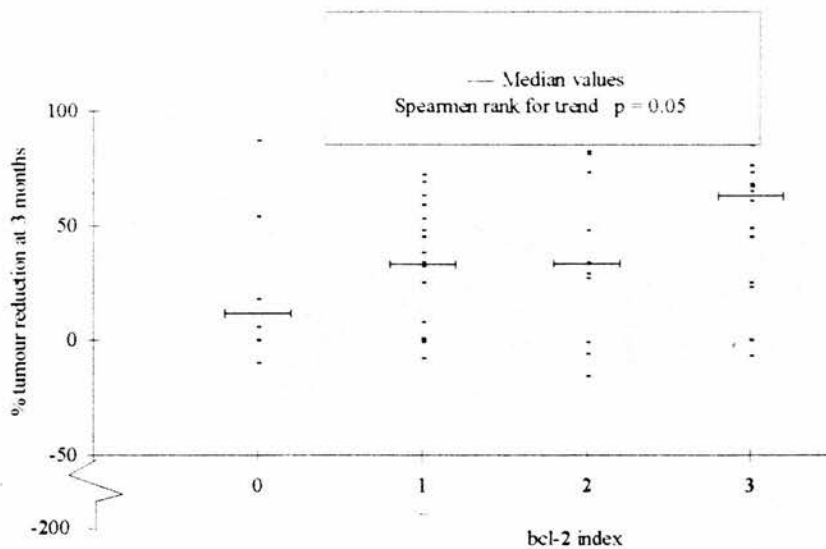


Figure 2.3.3 Pre-treatment bcl-2 index and tumour response after 3 months tamoxifen therapy

Furthermore there is a correlation between the change in bcl-2 expression and response, with the more significant reductions in expression being associated with better responses to three months' tamoxifen ($z=1.96$, $p < 0.05$) - see figure 2.3.4.

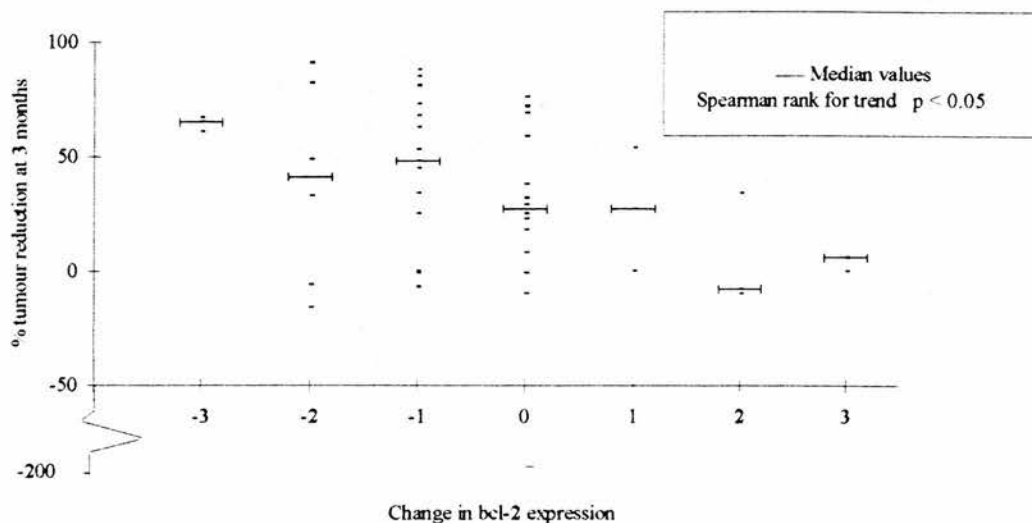


Figure 2.3.4 Tumour response and change in bcl-2 expression after three months' tamoxifen therapy

2.4. Apoptosis

Morphologically apoptotic cells were infrequent in the clinical breast cancers. The mean number of tumour cells examined was 1687, with a range of 1000 - 4100 in the pre-treatment biopsies and 584 - 3848 (with 5 tumours having < 1000 cells) in the post-treatment sections. The apoptotic indices before and after treatment are shown in table 2.4., and there is no significant difference between them.

| | minimum | mean \pm s.e. | maximum |
|-----------------------|---------|------------------|---------|
| pre-treatment | 0% | 0.45% \pm 0.04 | 1.5% |
| post-treatment | 0% | 0.37% \pm 0.04 | 1.6% |

Table 2.4. Apoptotic indices before and after three months' tamoxifen therapy.

There was a statistically significant correlation between the apoptotic index and the expression of bcl-2, as shown in figure 2.4., with the tumours expressing high levels of bcl-2 having fewer apoptotic cells ($z = -2.1$, $p < 0.05$). When considering the pre- and post-treatment tumours separately, this relationship only held true for the former group of tumours ($z = -1.95$, $p = 0.05$ & $z = -0.45$, $p > 0.1$ respectively), as shown in figure 2.4.2.

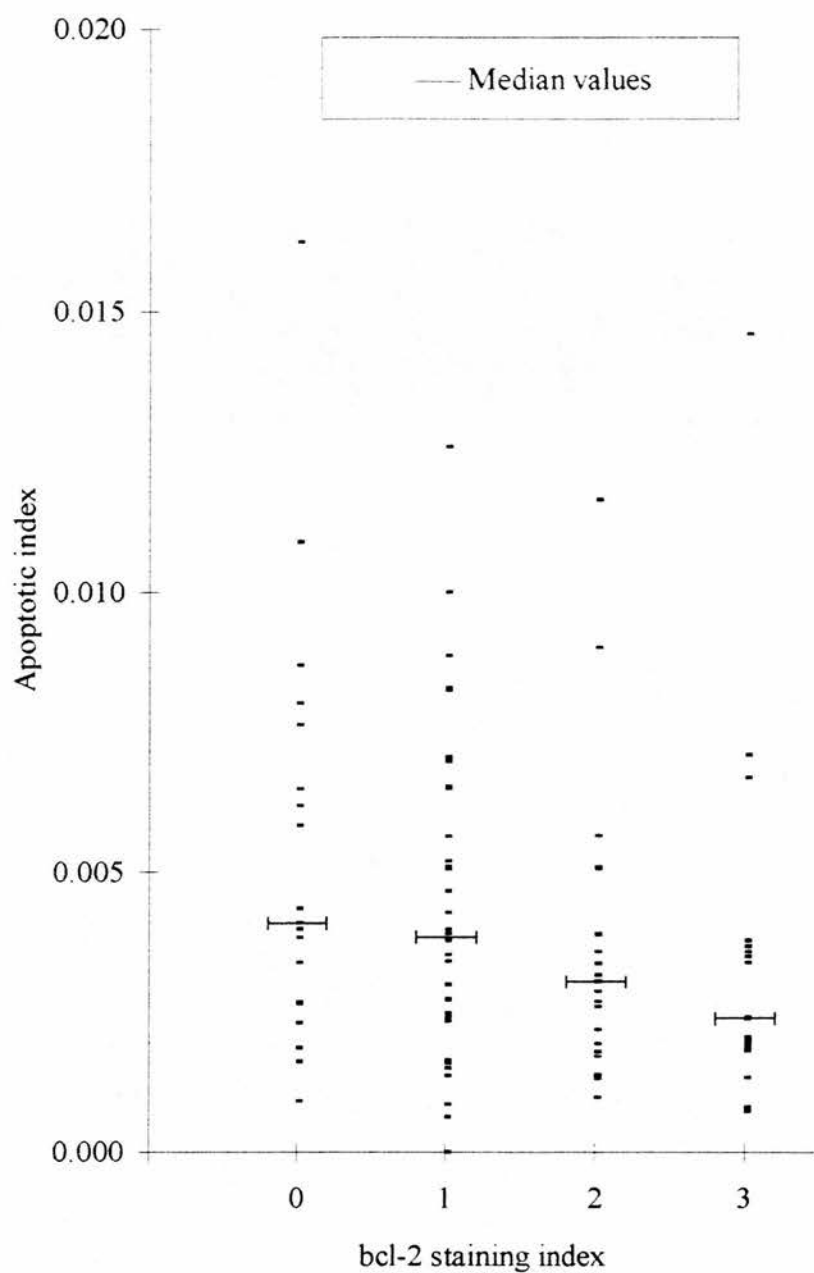


Figure 2.4.1 Apoptotic index & bcl-2 for all pre- and post-treatment tumours

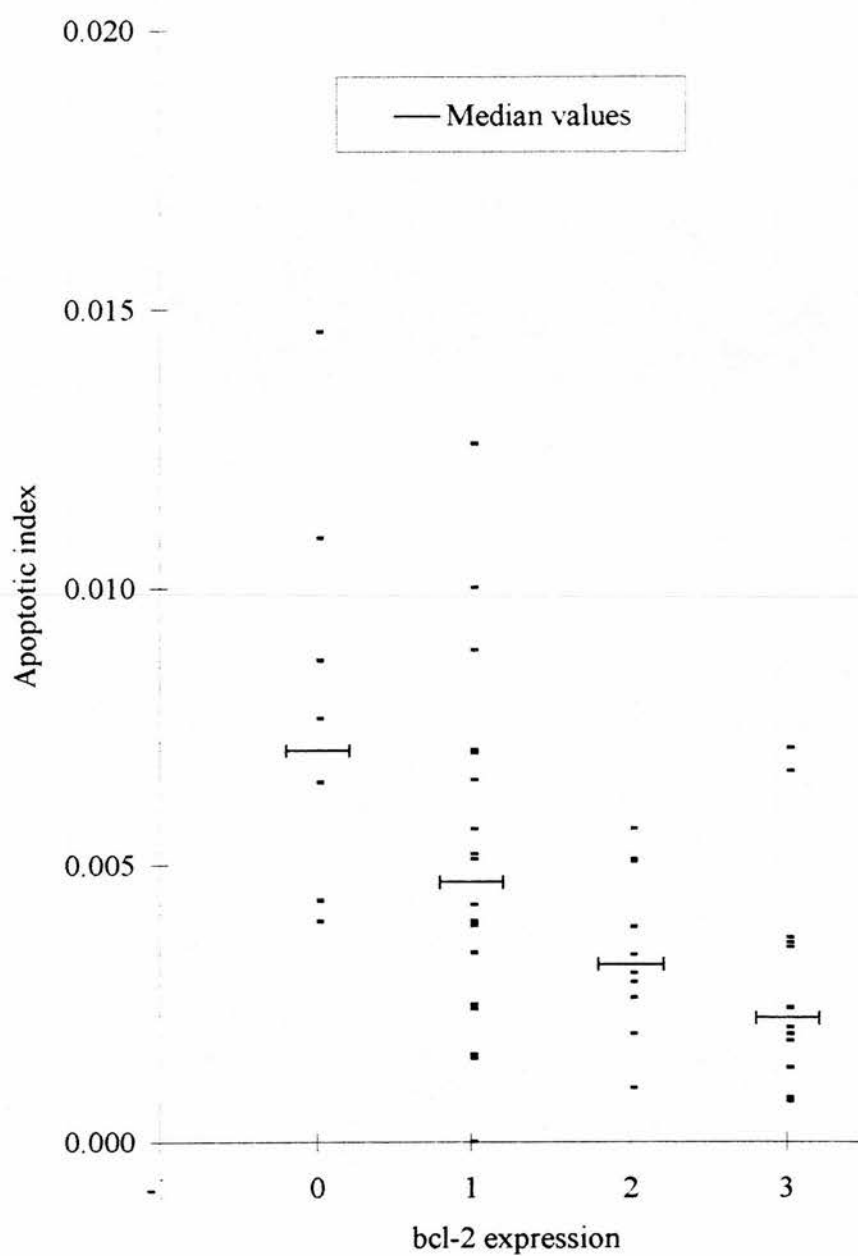


Figure 2.4.2 Pre-treatment Apoptotic index and bcl-2 expression

For the changes in apoptosis, there were no correlations with response, or ER concentration. However, figure 2.4.3 shows the significant correlation between the change in apoptosis and the change in bcl-2 expression ($z = -2.66$, $p < 0.01$), which itself was also correlated with the initial apoptotic index ($z = -2.88$, $p < 0.005$) (figure 2.4.4).

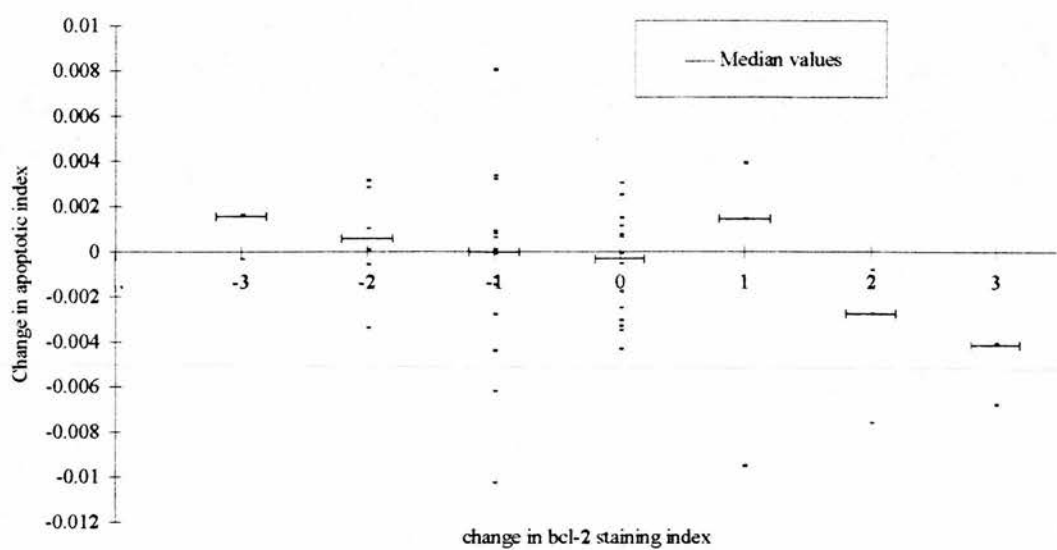


Figure 2.4.3 Change in apoptotic index and change in bcl-2 staining index

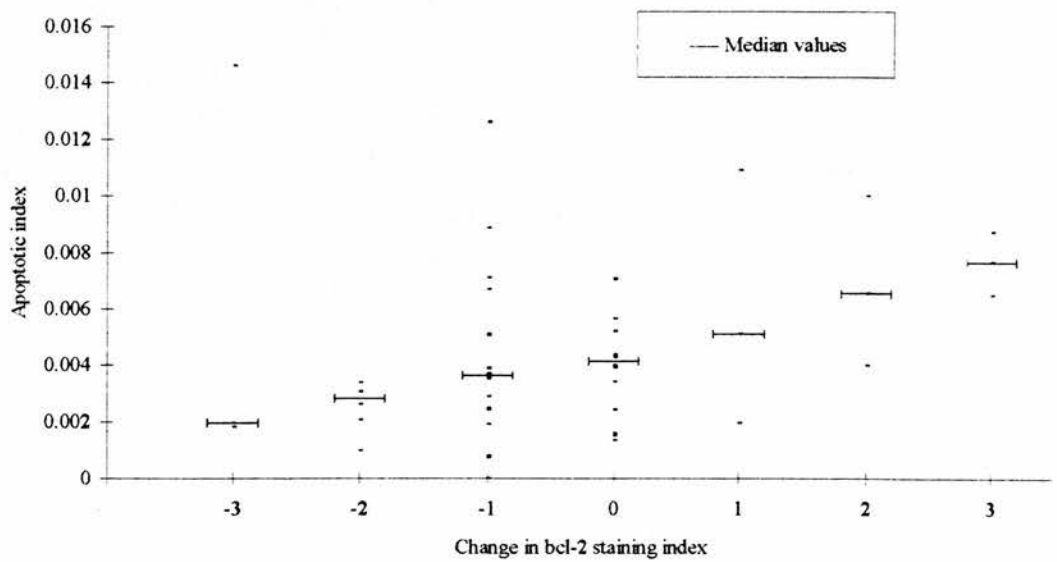


Figure 2.4.4. Change in bcl-2 staining index and initial apoptotic index

2.5. Mitosis

Mitotic figures were less frequent than apoptotic cells, but nevertheless there was a clear decrease in mitotic activity after 3 months' therapy ($W = 2850$, $p = 0.025$), as shown in table 2.5 and figure 2.5.1.

| | minimum | mean \pm s.e. | maximum |
|----------------|---------|------------------|---------|
| pre-treatment | 0% | 0.18% \pm 0.03 | 1.1% |
| post-treatment | 0% | 0.10% \pm 0.02 | 0.6% |

Table 2.5.1 Mitotic indices before and after three months' tamoxifen therapy.

Although the ER concentration is not associated before treatment with the mitotic index, it is significantly correlated with the mitotic index after three months' tamoxifen ($r = 0.34$, $p < 0.02$) ; the higher the initial ER, the higher the residual mitotic index after three months' tamoxifen, as shown in figure 2.5.2. Furthermore there are no correlations between the post-treatment ER concentration and the mitotic index before or after three months' tamoxifen therapy.

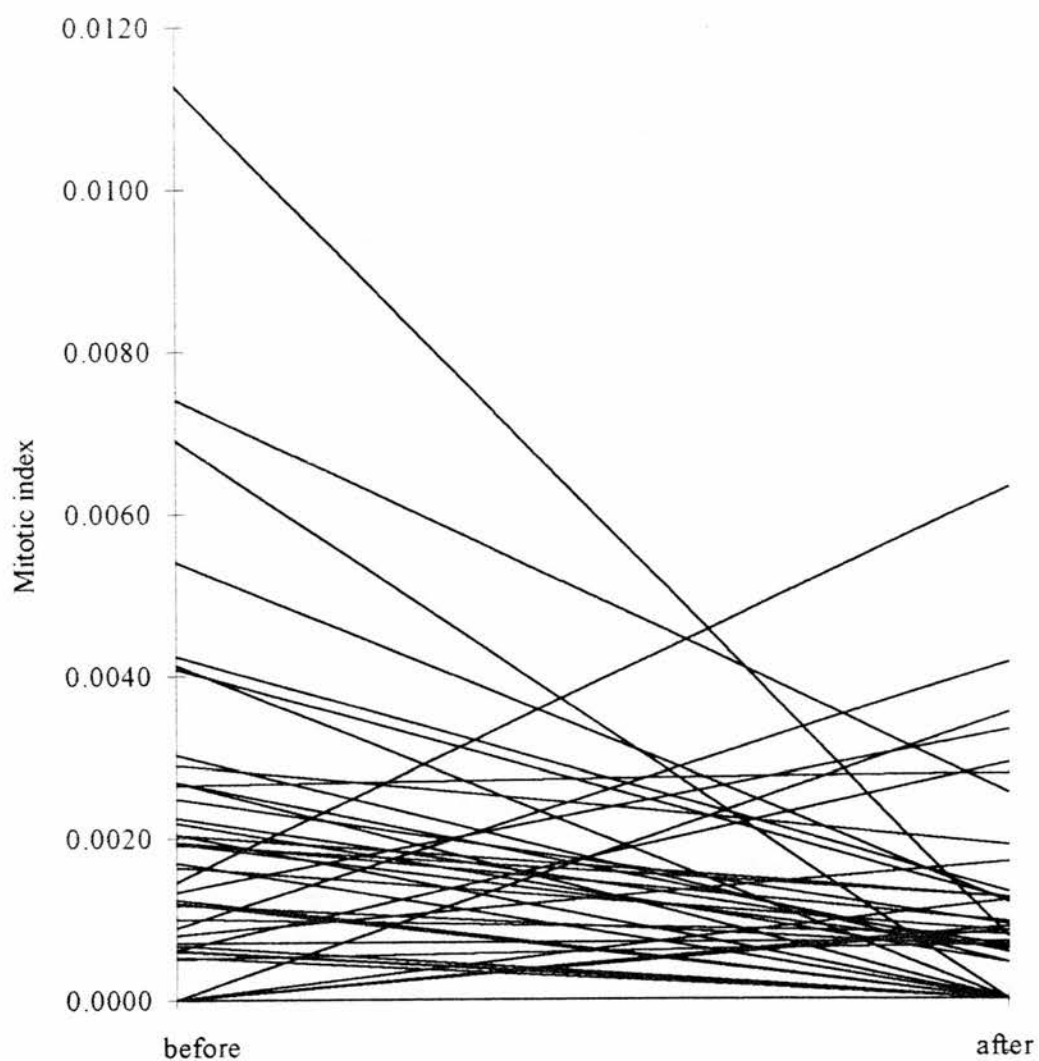


Figure 2.5.1. Mitotic indices before and after three months' tamoxifen therapy

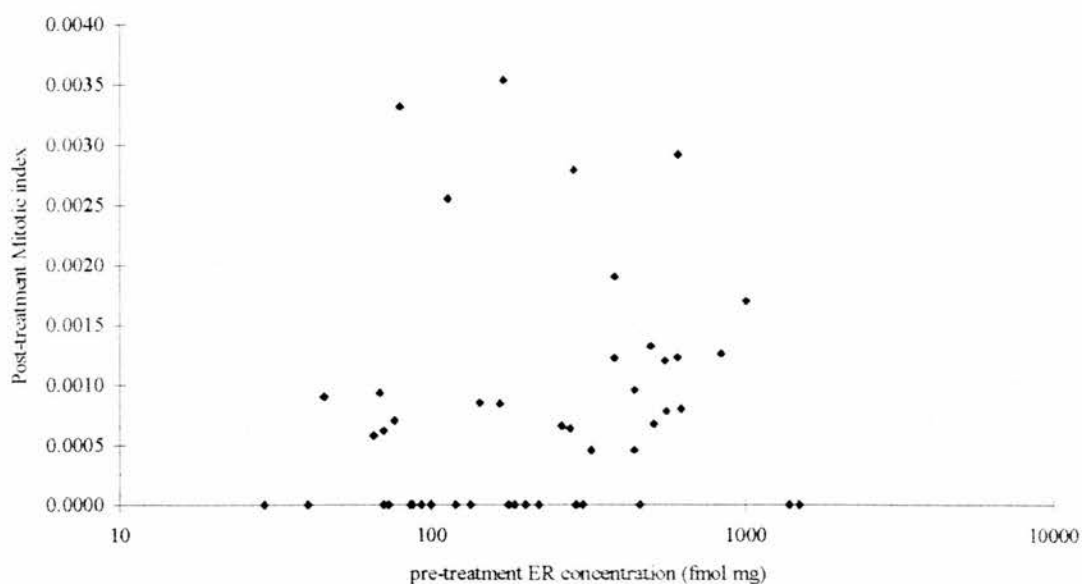


Figure 2.5.2 Pre-treatment ER concentration and post-treatment Mitotic index

Furthermore, although the pre- and post- treatment mitotic indices did not correlate with response as a continuous variable, there were significantly more non-responders with a 0 mitotic index after treatment ($p < 0.03$, Fisher's exact test), as shown in table 2.5.2, although there are no differences in the pre-treatment mitotic indices of responders and non-responders. There were also no differences in the change in mitosis with treatment between responding and non-responding tumours.

| | post Mitotic index = 0 | post Mitotic index > 0 |
|-----------------------|------------------------|------------------------|
| Responders | 10 | 25 |
| Non-responders | 10 | 5 |

Table 2.5.2. Post-treatment mitotic index and response

That this is not just the result of arbitrarily picking a zero mitotic index as the cut-off, is indicated by the observation that the post-treatment mitotic indices are in general lower in the non-responders ($W = 289$, $p < 0.05$), as shown in figure 2.5.3. Furthermore, although there are no differences in the apoptotic indices between the two groups of tumours with a zero post-treatment mitotic index, figure 2.5.4 shows that overall the apoptotic index is higher after treatment in the tumours which still have detectable mitoses ($W = 897.5$, $p < 0.01$).

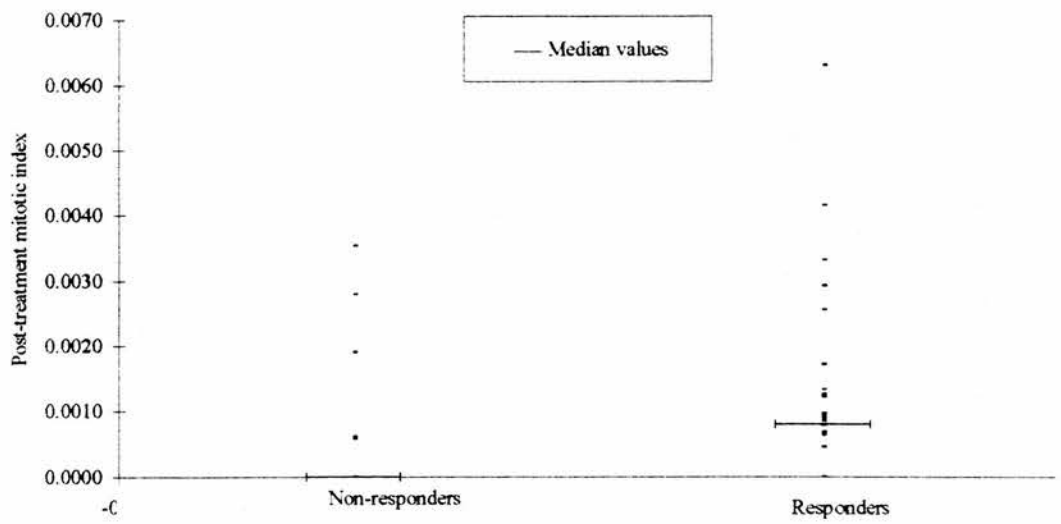


Figure 2.5.3 Mitotic index and response after three months' tamoxifen

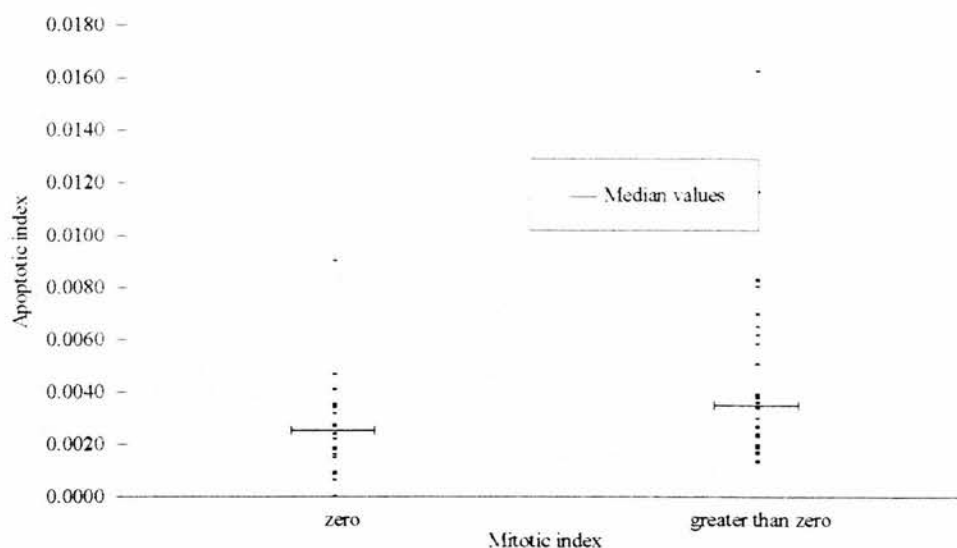


Figure 2.5.4 Apoptotic and Mitotic indices after three months' tamoxifen

There is in fact a more general positive relationship between the apoptotic and mitotic indices, which is true for tumours both before ($r = 0.375$, $p < 0.01$) and after three ($r = 0.380$, $p < 0.01$) months' tamoxifen. This is illustrated in figures 2.5.5 and 2.5.6, where, if it is assumed that the mitotic rate is the independent variable, it can be seen that the nature of the relationship is changed by the therapy, with the steeper line occurring in the tumours after therapy. In contrast if the apoptotic index is considered to be the independent variable (which is less biologically likely as without proliferation there would be no tumour cells to undergo apoptosis), then the regression lines are almost identical before and after treatment (data not shown).

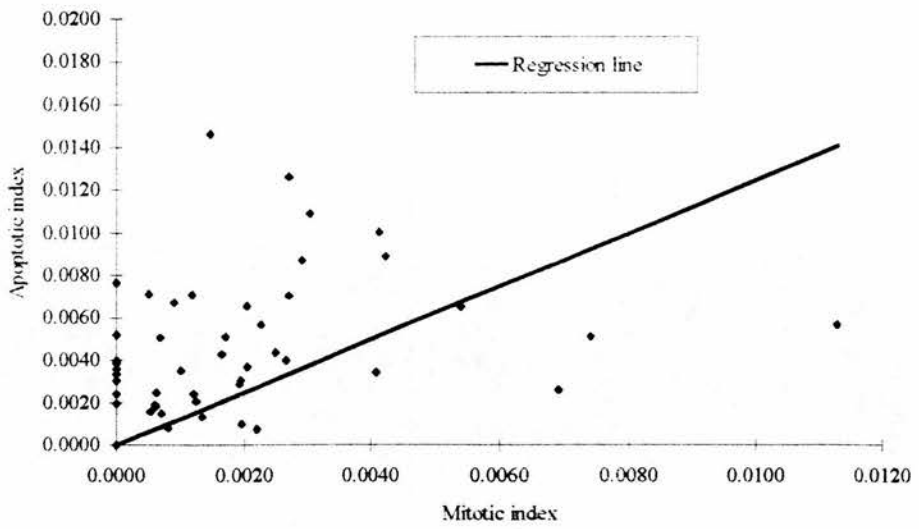


Figure 2.5.5 Correlation between Apoptotic and Mitotic indices before tamoxifen treatment
(with Regression line forced through the origin)

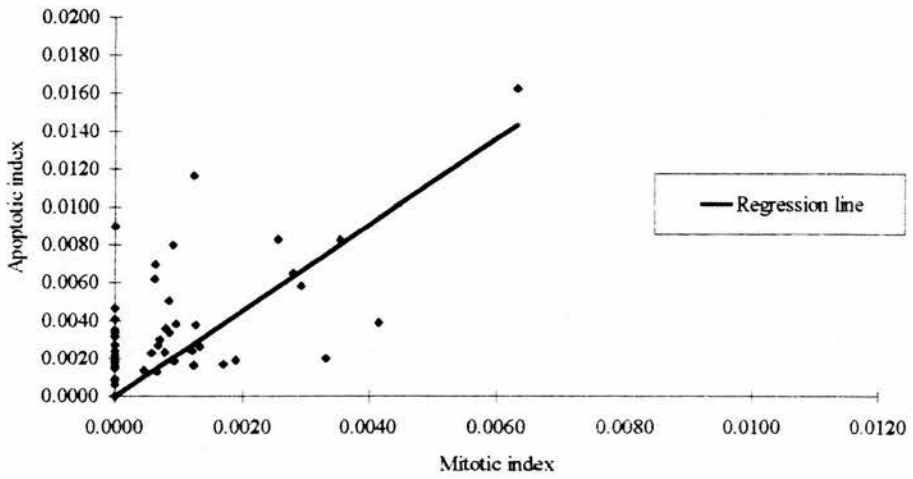


Figure 2.5.6. Correlation between Apoptotic and Mitotic indices after tamoxifen therapy (with
Regression line forced through the origin)

2.6 Apoptotic:Mitotic ratio

Since tumour growth is a reflection of both cell division and death, one way of combining the histological assessments of apoptosis and mitosis is the ratio of the number of apoptotic cells : number of mitotic cells. In those tumours in which no mitotic figures were seen, the ratio is underestimated as being equal to the total number of apoptotic figures seen. (This situation arose in 13 pre-treatment samples and 20 post-treatment cases.) As table 2.6. suggests, there is no significant difference between the values before and after treatment, which is also true when considering the sub-groups of tumours which have responded or not.

| | minimum | mean \pm s.e. | maximum |
|----------------|---------|-----------------|---------|
| pre-treatment | 0.333 | 3.5 ± 0.4 | 14 |
| responders | 0.3 | 2.5 ± 0.5 | 14 |
| non-responders | 1.5 | 3.0 ± 0.8 | 8 |
| post-treatment | 0.6 | 3.5 ± 0.4 | 11 |
| responders | 0.6 | 3.0 ± 0.4 | 11 |
| non-responders | 1.0 | 2.2 ± 0.8 | 10 |

Table 2.6. Apoptotic:Mitotic ratio before and after three months' tamoxifen therapy.

For correlations with other data, it is the normally distributed log-transformed apoptotic:mitotic ratio that is considered. There are no significant correlations between the apoptotic:mitotic ratio and any of the previously presented data (apart from the synchronous apoptotic or mitotic ratios). For the change in the (log of) the apoptotic:mitotic ratio, there is however a trend towards significance with the response ($r = 0.314$, $p < 0.05$), as shown in figure 2.6.1.

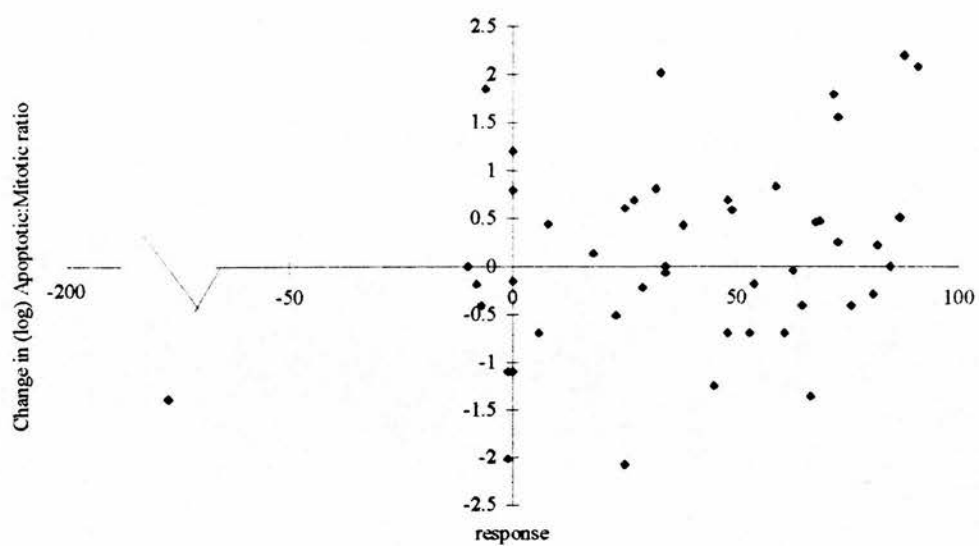


Figure 2.6.1 Change in Apoptotic:Mitotic ratio and response to three months' tamoxifen therapy

2.7. Discussion

The data (figure 2.2.2) confirm that the level of ER predicts for the degree of response to tamoxifen as has been previously shown (Brooks et al(1980); Gaskell et al(1992a); Osborne et al(1980)). Most previous studies that have observed this relation have included significant numbers of ER-negative tumours (Allan et al(1985); Nicholson et al(1991)), which have a low response rate to primary tamoxifen therapy (Anderson et al(1989)). The relationship between the degree of ER positivity and the response to primary tamoxifen has also been noted before (Gaskell et al(1992a); Osborne et al(1980)), although not with both parameters as continuous variables. The overall response rate seen at three months, namely 70%, compares favourably with other studies (Allan et al(1985)), although a non-standard definition of response was used. However the formal UICC criteria include a repeat assessment of response a month later, and that would not have been possible as the patients proceeded to definitive loco-regional surgery at three months.

The change in ER with treatment, as shown in figure 2.2.1 and table 2.2 is also consistent with previous literature (Allegra et al(1980); Lumsden et al(1997); Taylor et al(1982)). The observation in figure 2.2.3 that the degree of response and the fall in ER are positively correlated has not been previously reported, but is consistent with effective anti-oestrogenic therapy, but also the possibility that those tumours that manifested a greater response had less tumour in which to measure the residual ER. This latter possibility should however be at least in part overcome by the use of an ER corrected for the weight of available cytosolic protein, and the data therefore suggest that effective tamoxifen therapy might be a more potent inhibitor of ER expression than ineffective therapy. However as a means to predict tamoxifen sensitivity, the change in ER is not sufficiently specific.

The positive association between bcl-2 positivity and ER seen in figure 2.3.2 has been previously recognised (Gee et al(1994); Johnston et al(1994); Krajewski et al(1995)). Furthermore a relationship between bcl-2 expression and response to tamoxifen therapy, as suggested by figure 2.3.3 has also been previously reported (Gee et al(1994)). However in that study a significant positive correlation was found when tumours were classified as either positive or negative for both markers. A correlation was also reported for degrees of positivity, but the statistics were invalid (using the χ^2 test with small numbers). Performing more appropriate tests, such as a non-parametric trend

test, the result remains significant, but only because of 12 progressing ER and bcl-2 negative tumours : there is no relationship for the remaining 28 ER-positive tumours. There are thus no previous data reporting a significant relationship between bcl-2 expression and response in ER-positive tumours, but it is none-the-less to be expected as bcl-2 is induced by oestrogen (Teixeira et al(1995)), and the data indeed suggest that it may be a marker for a hormone-sensitive tumour (as opposed to one that merely reflects the oestrogen receptor).

Furthermore, consistent with this relation between bcl-2 and hormone-sensitivity, is the fall in expression seen over the three month period, as tamoxifen can inhibit its oestrogen-induced expression in vitro (Wang et al(1995)). However there has been only one previous study comparing bcl-2 levels before and after tamoxifen therapy, in which a significant increase was reported in 11 tumours of unknown ER and tamoxifen-sensitivity (Johnston et al(1994)). However in the current study 18% (9/50) of the tumours showed increased expression after three months' tamoxifen therapy (see figure 2.3.1), but 5 of these did not respond ($p = 0.1$, Fisher's exact test). Hence the previously reported results (Johnston et al(1994)) would not be inconsistent with a majority of the tumours being tamoxifen insensitive. The correlation between the degree of fall in bcl-2 expression and the degree of response at three months (see figure 2.3.4) suggests that effective anti-oestrogen therapy inhibits the expression of bcl-2 in ER-positive tumours.

The level of apoptosis detected is broadly similar to the only other published study which also failed to show any overall change with tamoxifen treatment (Dowsett et al(1995)). Another study of apoptosis in breast cancer has reported the higher rate of around 1% (Vandeschepop et al(1996)) ; but this was not confined to ER-positive tumours, and may have included ER-negative tumours. As grade correlates with proliferation (Haerslev et al(1996); Pierga et al(1996)), which in turn correlates with apoptosis (Lipponen et al(1994)), the inclusion of ER negative tumours could result in a higher overall rate of apoptosis. The inverse correlation between apoptosis and the level of bcl-2 expression (see figure 2.4.1) is not unexpected, as bcl-2 has an important role in inhibiting apoptosis (Hockenbery et al(1990)), and has been noted previously (Sierra et al(1995)), using an indirect method which, because it was based on the detection of fragmented DNA, may also detect necrotic cells (Gold et al(1994)), and necrosis is associated with high-grade tumours (Clayton, (1991)) and ER negative tumours (Fisher et al(1981)) which are themselves less likely to express bcl-2 (Lipponen

et al(1995); Sierra et al(1995)). There are two previous reports on the relationship between bcl-2 expression and apoptosis as assessed morphologically. One (Chan et al(1993)) observed the inverse association between bcl-2 and apoptosis (as in the present study), particularly in tumours with a low proliferative rate, but it has not been formally published, partly due to problems with inter-observer discrepancies (A Hanby, personal communication). The other (Lipponen et al(1995)), although finding a weak trend, reported in contrast highly significant negative correlations between bcl-2 expression and markers of proliferation. What is not immediately apparent is the reason that the relationship between apoptosis and bcl-2, apparent before therapy (figure 2.4.2) is not maintained after three months' tamoxifen, and this will be discussed further (vide infra).

The lack of any significant correlations between the change in apoptosis, and response or ER level are perhaps not surprising, given that overall there was no change in the level of programmed cell death after three months' tamoxifen. However there was a relationship with the change in bcl-2 expression (figure 2.4.3), which might be anticipated as the latter inhibits apoptosis in many cell systems. The relationship is not as strong as that between the initial level of apoptosis and change in bcl-2, suggesting that factors other than the level of bcl-2 expression are also important in determining the rate at which tumour cells undergo apoptosis after three months' tamoxifen. The relation between change in bcl-2 expression and initial apoptotic index may be due to the relation between the apoptotic index and bcl-2, which itself is related to response, and thus (as in figure 2.3.4) to the change in bcl-2 expression.

The loss of a linear relationship between bcl-2 expression and apoptotic index with therapy could be due to variations in the degree of true endocrine-sensitivity, for which the best available indicator is the tumour response. When this is considered, it is found that the tumours with a fall in bcl-2 expression which responded have a rise in the apoptotic index that is significantly higher than the non-responders ($W = 52$, $p = 0.07$ for change in AI and $W = 48$, $p < 0.04$ for % change) (and see figure 2.7.1). Similarly, when considering only the responding tumours, then those whose bcl-2 expression falls have a significantly higher rise in the apoptotic index ($W = 447$, $p < 0.025$ for change in AI and $W = 442.5$, $p < 0.05$ for % change) as compared with those whose bcl-2 rises or remains unchanged. Thus effective tamoxifen therapy is associated both with a fall in bcl-2 expression and a rise in apoptosis, although whether one causes the other

remains unclear. In contrast there were no differences in the degree of reduction in mitotic activity between these subgroups.

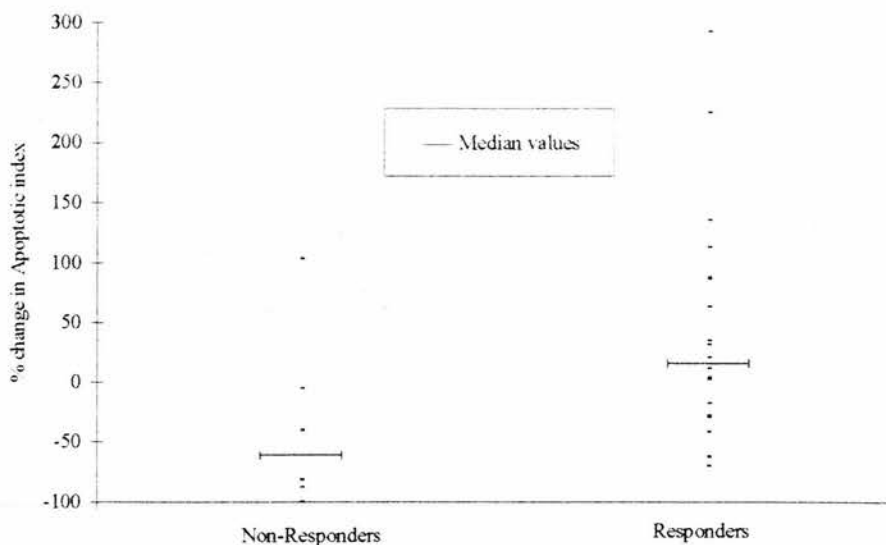


Figure 2.7.1. Percentage change in apoptotic index for tumours with a fall in bcl-2 expression after three months' tamoxifen

The reported mitotic index is consistent with other reports for clinical breast cancer. There are none which record the data in the same manner as this study ; but a larger study of over 300 tumours recorded an average of 0.47 mitosis per high-power field (Clayton, (1991)) (corresponding to an index of around 0.2%, slightly lower than what was noted in this study) ; another smaller study noted that bcl-2 positive tumours in particular had a mitotic index of less than 0.6% (Chan et al(1993)). The fall in mitotic index with treatment is also consistent with previous studies (Clarke et al(1993); Johnston et al(1994)) (although both studies used Ki-67 labelling rather than mitotic index). It is of interest that although there is no relation before treatment between the ER concentration of the tumour and its proliferative fraction, after three months' tamoxifen it would seem that the tumours with higher ER values have more proliferation.

The observation that there were significantly more non-responding tumours with a zero post-treatment mitotic index would seem at first to be at odds with data that demonstrate tamoxifen's anti-proliferative effects, and remains incompletely explained as there were no significant differences in apoptosis between the responding and non-

responding tumours in the sub-group of tumours with no detectable mitoses after treatment. However there were significantly more apoptoses in the tumours with detectable mitoses after treatment, and mindful of the fact that most (13/15) of the tumours classified as non-responders were static, one has a majority (8/13) of static tumours with no proliferation (and thus less apoptosis) after tamoxifen therapy. Thus they may be non-responders simply because they have minimal mitotic and apoptotic activity, and thus any change in volume over a three month period is too small to be reliably detected.

These data also confirm that there is a positive relation between the proportion of cells in mitosis and undergoing apoptosis, both before and after three months' tamoxifen therapy (figures 2.5.5 & 2.5.6). This has been previously reported for breast cancers (Lipponen et al(1994)), low grade Non-Hodgkin's lymphomas (Czader et al(1996)) and bladder tumours (Lipponen et al(1994)), and although the methodologies used were slightly different the relative incidences are comparable. However figure 2.5.6 suggests that nature of this relationship is altered by the treatment, which has not previously been noted.

What then is the relation between these two effects of tamoxifen - reduction in bcl-2 expression (and in some cases a concomitant increase in apoptosis) and its anti-proliferative action ? The ratio of apoptosis to mitosis has been applied in studies of xenografts (Arends et al(1994)). As can be seen in table 2.6., the ratio does not change after three months' tamoxifen therapy, and did not correlate with changes in other markers, apart from a trend towards significance with tumour response, shown in figure 2.6.1. For many tumours there was only a small change in the ratio, but the subsequent clinical behaviour of these tumours remains unknown, and experience with other patients managed similarly, but who declined surgery at three months, reveals that some of those whose tumours have responded may subsequently progress, whereas others will undergo further regression on continued exposure to tamoxifen (unpublished observations). Hence it could be conjectured that the tumours which manifested a fall in the ratio at three months might have subsequently remained static or progressed if they had not undergone surgery. Consistent with this are the "outliers" in the series : the tumours with the largest increases are all responders, and the two progressing tumours both had a fall in the ratio, although in one case it was indefinable as no apoptotic or mitotic figures were seen in the post-treatment specimen.

An alternative method to study the relation between the observed effects of tamoxifen on both bcl-2 and proliferation is to look at sub-groups of tumours, although in this small study such analyses must be interpreted with caution. It is interesting therefore to note that the effect of tamoxifen on the apoptotic indices of the tumours with a low initial apoptotic:mitotic ratio is significantly different ($\chi^2 = 6.77$, $p < 0.01$) as compared with those with higher initial values, with a more of the tumours with an initially low apoptotic:mitotic ratio manifesting a rise in apoptosis three months' later, whilst no such differences are seen for mitotic index (see table 2.7.1).

| initial ln(A/M) | Change in Apoptotic index | | | Change in Mitotic index | | |
|-----------------|---------------------------|---|---------|-------------------------|---|---------|
| | ↓ > 20% | = | ↑ > 20% | ↓ > 20% | = | ↑ > 20% |
| < 0.5 | 3 | 1 | 8 | 9 | 1 | 2 |
| > 0.5 | 21 | 8 | 8 | 20 | 1 | 6 |

Table 2.7.1. Effect of three months' tamoxifen therapy on the apoptotic and mitotic indices according to their initial relative levels.

Further examination of the data shows that there are significant differences in the pre- and post-treatment apoptotic and mitotic indices for these two groups (see table 2.7.2.) The differences between the mitotic indices for the two groups of tumours are significant (see table 2.7.2) ($W = 459$, $p < 0.001$ & $W = 413$, $p < 0.02$ for the pre- and post- values respectively). In contrast the difference between the apoptotic indices is not significant, although there is a possible trend towards it being higher pre-treatment in the group with the higher ratio ($W = 223$, $p = 0.06$). Thus the division into two groups of tumours according to their initial apoptotic:mitotic ratio appears to reflect the differences in their initial mitotic rates, yet the contrasting effects of three months' tamoxifen are more apparent on the level of apoptosis.

| initial ln(A/M) | Median Apoptotic index | | Median Mitotic index | |
|-----------------|------------------------|--------|----------------------|--------|
| | pre- | post- | pre- | post- |
| < 0.5 (n = 12) | 0.0030 | 0.0026 | 0.0025 | 0.0012 |
| > 0.5 (n = 37) | 0.0040 | 0.0027 | 0.0007 | 0.002 |

Table 2.7.2. Median apoptotic and mitotic indices for tumours classified by their initial apoptotic:mitotic ratio.

When considering the bcl-2 scores for these two groups of tumours, there were no differences in the absolute values or the change in bcl-2 scores. However there is a

significant difference in the response to tamoxifen ($W = 409$, $p < 0.02$), as shown in table 2.7.3.

| initial ln(A/M) | Median response | non-responders |
|-----------------|-----------------|--------------------------|
| < 0.5 | 70% | 2/12 (17%) (both static) |
| > 0.5 | 32% | 13/37 (35%) |

Table 2.7.3. Response for tumours classified by their initial apoptotic:mitotic ratio.

This suggests that there may be two distinct phenotypes of tumours - those with a low initial apoptotic:mitotic ratio, in which tamoxifen appears to induce apoptosis, and a measurable tumour regression, and a larger group of tumours with a higher ratio (due in large part to a lower proliferation rate) which are much less likely to respond with either an increase in apoptosis or tumour regression. However in contrast, the anti-proliferative effect of tamoxifen is indistinguishable between these two groups, consistent with previous reports of a reduction in proliferative fraction (as measured by MIB-1) irrespective of the level of ER (Clarke et al(1993)). The reasons for this distinction remain unclear, but the association between a zero post-treatment mitotic index and lack of response, (as shown in figure 2.5.3 and table 2.5.2) is relevant, as 19/20 of such tumours are in the high apoptotic:mitotic ratio group (a highly significant difference - $p < 0.02$, Fisher's exact test), and their response is not different from the other 18 tumours in the same group. A tumour with minimal proliferative activity after three months' exposure to tamoxifen will have less apoptosis (as the relationship between these two processes seems fundamental), and is therefore likely to be static. This was the case for the majority of non-responders in this series. There is however no certain evidence that these differences in response are due to different levels of apoptosis after three months' therapy.

Thus the data presented here confirm previous observations, but do not answer the question of whether or not bcl-2 expression inhibits apoptosis or is merely co-expressed in a cell with a low apoptotic rate for other reasons. In favour of the former argument is the observation that the change in bcl-2 expression is inversely related to the change in apoptosis - with a decrease in the former being associated with a fall in bcl-2 level. On the other hand, there is considerable evidence to suggest the latter explanation. There are reports of strong inverse relationships between bcl-2 expression and markers of high proliferation (Binder et al(1995); Lipponen et al(1995)) as well as the observed relationship between the apoptotic and mitotic indices, which are strongly related in

both treated and untreated tumours (see figure 2.5.5 & 2.5.6). Hence we have a putative phenotype of a high ER, high bcl-2, low mitotic (and thus low apoptotic) tumour, which is more sensitive to tamoxifen. One might therefore imagine that as effective tamoxifen treatment reduces the level of bcl-2 expression, there would be a concomitant increase in the level of apoptosis. However the inverse relation between bcl-2 expression and apoptosis is not as apparent after three months' tamoxifen therapy, and therefore it may be that changes in apoptosis and bcl-2 expression are both reflections of an anti-oestrogenic effect of tamoxifen, but are not directly linked.

3. ZR-75-1 Xenografts

ZR-75-1 human breast cancer xenografts were, as explained in the methods, derived from three separate passages (Nos. 25, 28 & 29). There were minor differences in the results for the tumours from these different passages, but none that alter the overall conclusions ; therefore all the results have been combined to simplify the data presented. This section will report data on groups of tumours removed at the different time points and treatment status. Results and the correlation with tumour growth for individual tumours will be covered subsequently in section 7.

3.1. Growth

The combined growth data are shown in figure 3.1.1. Prior to the insertion of the tamoxifen pellets there was no difference in the growth of the tumours allocated to the control and treatment groups. However although the control tumours continued to grow throughout the experiment, those tumours treated with tamoxifen regress, with a highly statistically significant difference in tumour volume occurring at day 14 and beyond ($p < 10^{-4}$ or less). However it should be noted that 4 of the untreated tumours that were excised on day 28 actually regressed between days 21 & 28, and their growth curves are shown separately in figure 3.1.1.

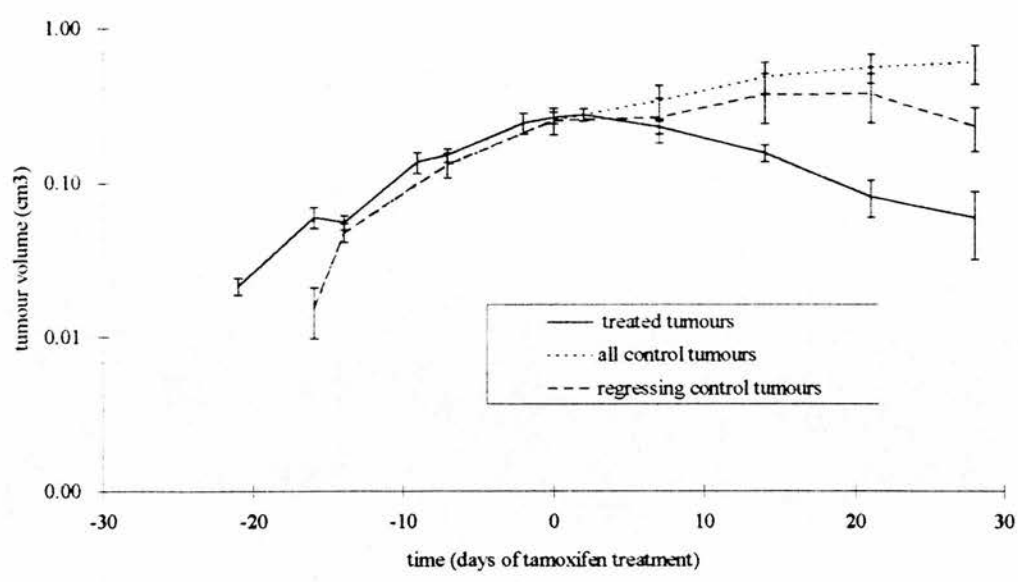


Figure 3.1.1. Growth of control and tamoxifen treated ZR-75-1 xenografts

3.2. Apoptosis & Mitosis

Apoptotic cells were infrequent in the ZR-75-1 xenografts, and as figure 3.2.1 illustrates, the incidence of apoptosis was higher in the tamoxifen treated xenografts on days 2 and 7. The values are statistically significantly different on day 2 both from the values in the day 0 untreated tumours ($W=98$, $p = 0.04$), and those in the day 28 controls ($W = 177$, $p = 0.01$). Furthermore there was a trend for the day 7 values to be higher than those in the day 28 controls ($W = 166$, $p < 0.07$). Combining the day 2 and 7 results together gives a significant difference as compared with the day 0 controls, and even more so when the day 0 and day 28 control values are combined ($W = 359$, $p = 0.005$). Thereafter the rate appears to fall, such that there are no differences between the values in the treated tumours on days 14 - 28 and either group of untreated tumours. Furthermore there were no differences in the apoptotic indices between the two groups of untreated tumours irrespective of whether or not they were regressing on day 28.

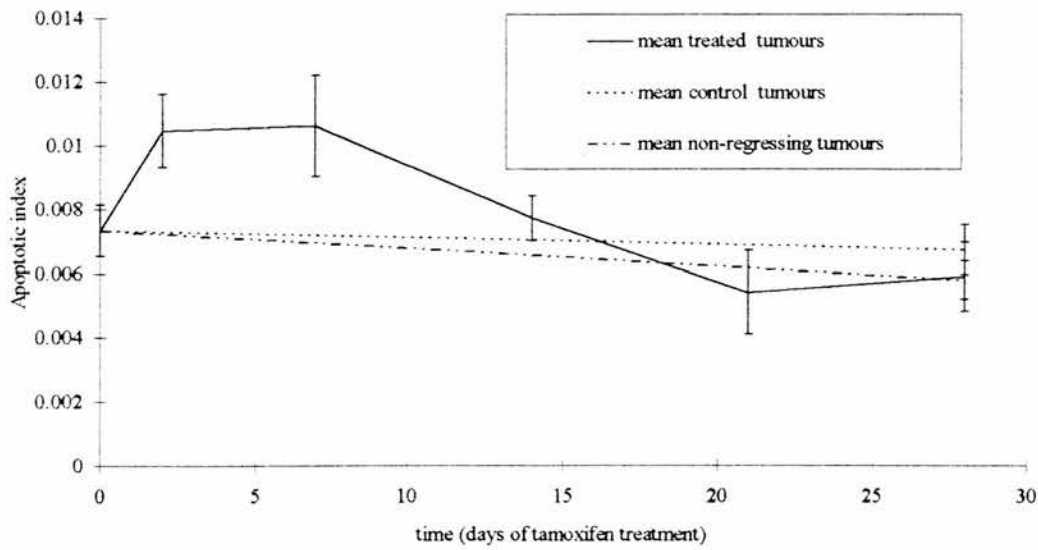


Figure 3.2.1 Apoptotic index in tamoxifen treated and control ZR-75-1 xenografts

Mitotic figures were less frequent than apoptotic figures, and the trends with time in the treated and untreated tumours are shown in figure 3.2.2, where it can be seen that the treated tumours have fewer mitotic figures than the day 0 untreated tumours, with statistically significant differences on days 7 ($W = 168$, $p < 0.03$), 21 ($W = 174$, $p = 0.002$) and 28 ($W = 322$, $p < 0.001$ respectively). On day 14 there is a trend in the same direction ($W = 153$, $p = 0.09$). Although there is no significant difference between the day 0 and day 28 control values, the latter tend to be lower ($W = 147$, $p < 0.08$), and thus when comparing the treated with the day 28 controls, a trend towards fewer mitotic figures is seen only on day 21 ($W = 134$, $p < 0.1$) and day 28 ($W = 256$, $p = 0.025$), conclusions not altered by including or excluding the regressing day 28 control tumours. If all the treated and control tumours are respectively combined, there are significantly fewer mitotic figures in the treated tumours ($W = 1330$, $p < 0.005$), with a highly significant downward trend over time for the mitotic index in the treated tumours ($z = -4.47$, $p < 0.0001$).

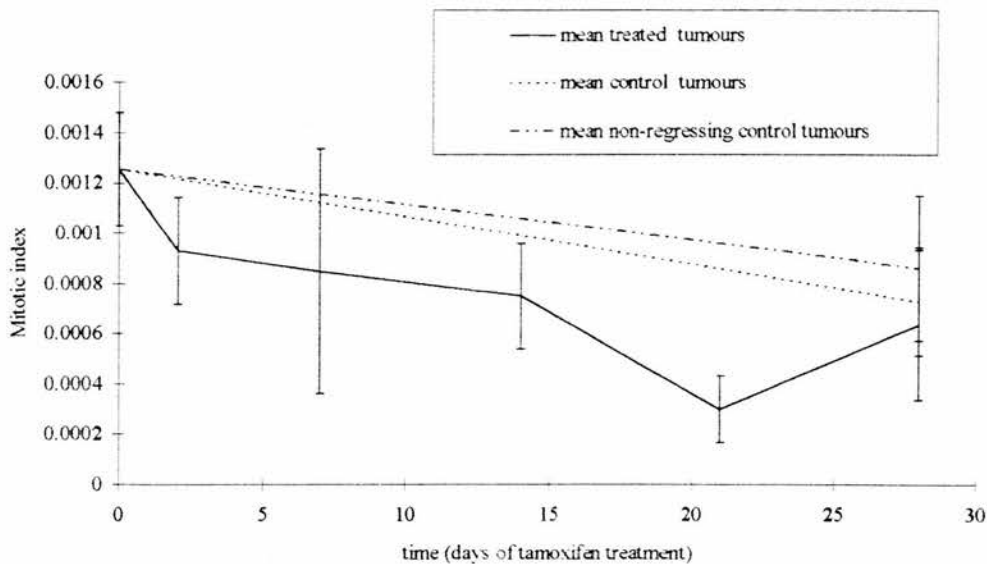


Figure 3.2.2. Mitotic index in tamoxifen treated and control ZR-75-1 xenografts

As the apoptotic/mitotic ratio is log-normally distributed, the two sample-t test is used for statistical considerations. As can be seen in figure 3.2.3, the ratio is generally higher in the treated tumours, as compared with the day 0 controls, although this is only statistically significant on days 2 ($p = 0.05$), and day 7 ($p < 0.005$). In contrast none of the values in the treated tumours are statistically significantly different from those in the day 28 untreated tumours. Although there is no significant difference between the values in the day 0 and day 28 controls, the ratios tend to be higher in the latter group, and if the regressing day 28 tumours are excluded, there is a trend towards a significant difference between the day 7 treated tumours and the day 28 controls ($p = 0.1$). Combining the results of the treated tumours from days 2 & 7, and the untreated tumours from days 0 & 28, there is a statistically significant difference ($p < 0.05$), which increases when the regressing day 28 control tumours are excluded ($p < 0.02$). Furthermore, over the 28 days of the experiment there is no significant trend for the apoptotic/mitotic ratio ($z = -0.88542$, $p > 0.25$), whereas there is over the first 7 days only ($z = 2.98$, $p < 0.005$); indeed between days 7 and 28 there is an equally statistically significant trend in the opposite direction ($z = -3.22$, $p < 0.002$).

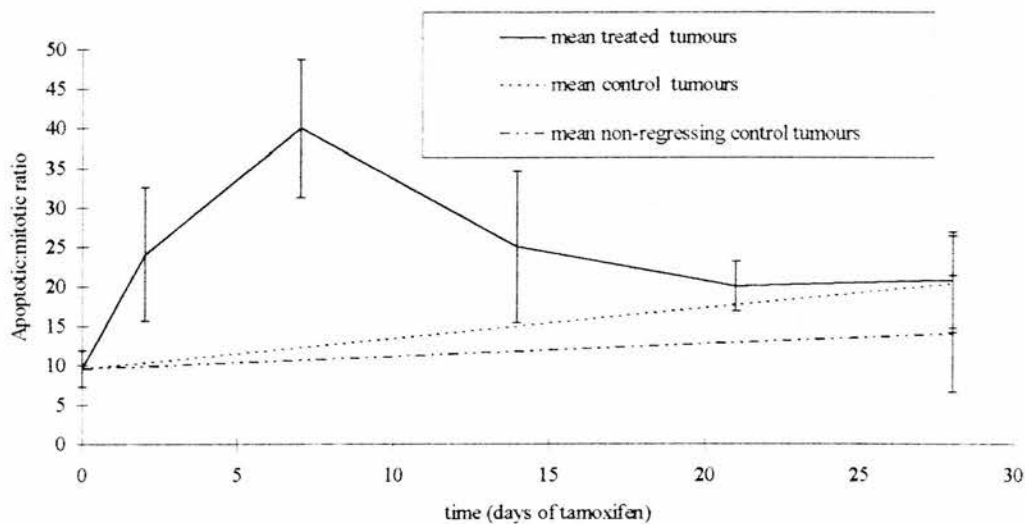


Figure 3.2.3. Apoptotic:Mitotic ratio in tamoxifen treated and control ZR-75-1 xenografts

3.3. MIB-1, bcl-2, p53 & c-erb-B2 expression

MIB-1 :

Figure 3.3.1 illustrates the data on the proportion of tumour cells expressing MIB-1. It can be seen that the mean value falls significantly with time in the tamoxifen treated tumours ($z = -3.73232$, $p < 0.0002$). At individual time points, as compared with the day 0 untreated tumours, the values are significantly different only on days 21 ($W = 160$, $p < 0.05$) and 28 ($W = 300$, $p < 0.01$). Furthermore there is no significant difference between the day 0 and day 28 control tumours.

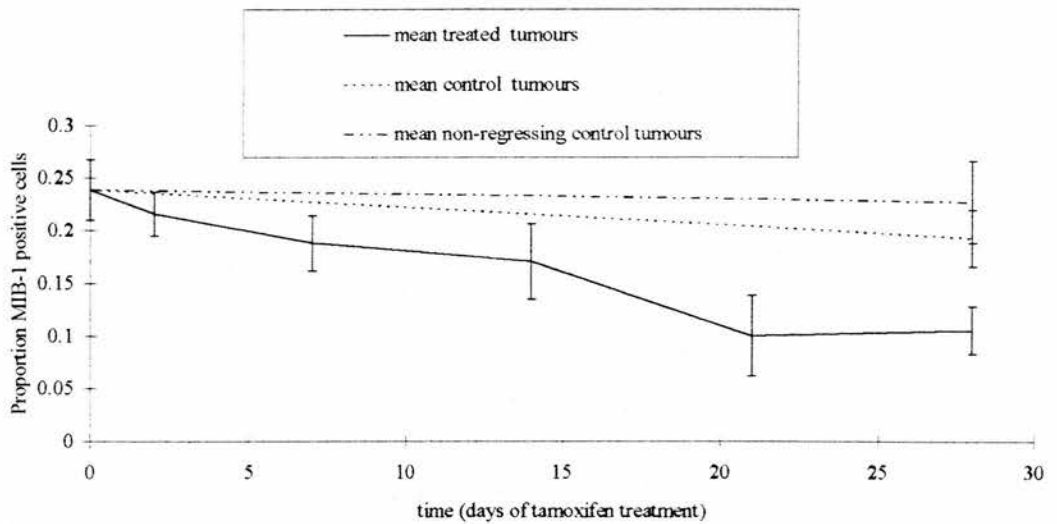


Figure 3.3.1. MIB-1 expression in tamoxifen treated and control ZR-75-1 xenografts

Bcl-2:

All ZR-75-1 tumour cells expressed bcl-2. There was no difference in intensity of staining at any time points between treated and untreated tumours.

p53 & c-erb-B2:

All ZR-75-1 tumour cells, irrespective of time points and treatment were consistently negative for both p53 and c-erb-B2.

3.4. Necrosis

The average proportion of each tumour section that was morphologically necrotic is shown in figure 3.4.1. and it can be seen that there is significantly less necrosis in the control tumours on day 28 as compared with day 0 ($W = 150$, $p < 0.05$). This is however no longer the case of the regressing control tumours are excluded. Furthermore, in the treated tumours, there are no differences as compared with the day 0 control tumours on days 2 & 7, but thereafter the necrotic proportion is statistically significantly less on days 14 ($W = 160$ $p < 0.05$), 21 ($W = 186$, $p = 0.0001$) and 28 ($W = 335$, $p = 0.0001$). This is confirmed by the trend test, which show a highly significant downwards trend over the 28 days ($z = -5.90141$, $p < 0.00001$).

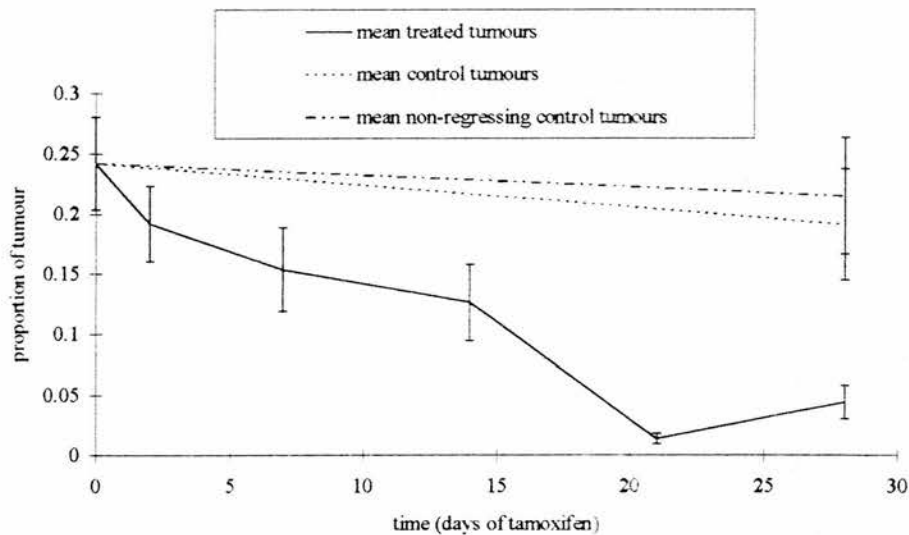


Figure 3.4.1. Necrotic proportion of tamoxifen treated and control ZR-75-1 xenografts

3.5. Vascular density

Some tumours from each passage were too small to be cut in half prior to fixation in paraformaldehyde. Thus the numbers available for cryostating are given in table 3.5.1. where it can be seen that the majority of the untreated tumours as well as most of the treated tumours from days 2 & 7 were available.

| day 0 | day 2 | day 7 | day 14 | day 21 & 28 | control day 28 |
|-------|-------|-------|--------|-------------|----------------|
| 6/11 | 11/12 | 9/12 | 5/11 | 0/38 | 6/10 |

Table 3.5.1 Numbers of ZR-75-1 xenografts with sufficient residual frozen material for cryostat sectioning.

The data on the vascular proportion of the tumours are shown in figure 3.5.1, where it can be seen that there appears to be less vasculature in the treated tumours. This is significant, as compared with either of the groups of untreated tumours, on days 2 ($W = 76$, $p < 0.05$) and 7 ($W = 69$, $p < 0.02$) but not on day 14. There is no difference between the day 0 and 28 control tumours, and there is no overall downwards trend between days 0 & 14 ($z = -1.13051$, $p > 0.2$). However it should be recalled that only 5/11 tumours excised on day 14 had sufficient residual tumour to permit cryostat sectioning, and they may therefore not be representative of the group as a whole. Combining all the treated tumours and comparing them with all the untreated tumours, a highly statistically significant difference was found between the two groups ($W=226$, $p < 0.0025$).

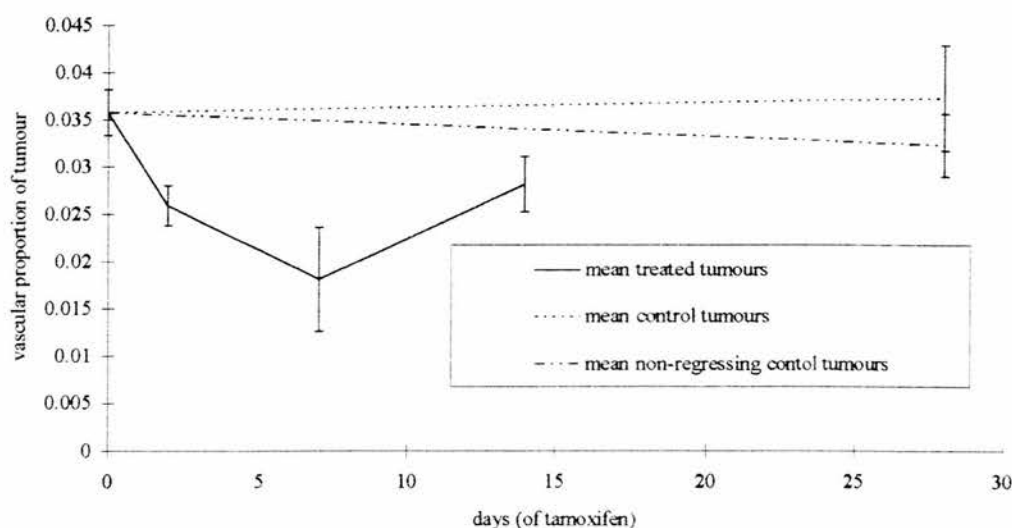


Figure 3.5.1 Vascular proportion of tamoxifen treated and control ZR-75-1 xenografts

3.6. Fine Needle Aspirates

Fine Needle aspirates were taken from all tumours, just prior to their excision. The intention was to determine whether or not FNAs could be used to detect the changes in tumour biology that were under consideration in this study. The samples were analysed on a flow cytometer, and therefore comparisons have been made between this data and those obtained by histological methods. However some FNAs were of insufficient quality for the cell cycle analysis programme to analyse. The following table summarises the available data :

| | treated tumours | | | | | control | |
|----------------|-----------------|----|----|----|----|---------|----|
| day | 2 | 7 | 14 | 21 | 28 | 0 | 28 |
| apoptosis | 12 | 11 | 5 | 0 | 18 | 7 | 6 |
| cell-cycle | 12 | 10 | 4 | 0 | 11 | 6 | 1 |
| No. of tumours | 12 | 12 | 11 | 11 | 27 | 12 | 10 |

Table 3.6.1 Numbers of tumours with material available for analysis on the flow cytometer.

The data on the flow cytometric analysis of the proportion of cells in the different phases of the cell cycle together with the fraction that are apoptotic will be presented.

3.6.1. Apoptosis

The available data for the flow cytometric analysis of apoptosis in the ZR-75-1 xenografts is shown in figure 3.6.1, and it suggests that the only difference between the treated and control values is on day 14, although there are no significant differences, nor any significant trend over the 28 days. It should be noted that the proportion of cells measured to be apoptotic by the flow cytometric method is very much higher than that when the morphological criteria were used on tissue sections, and this will be further discussed later.

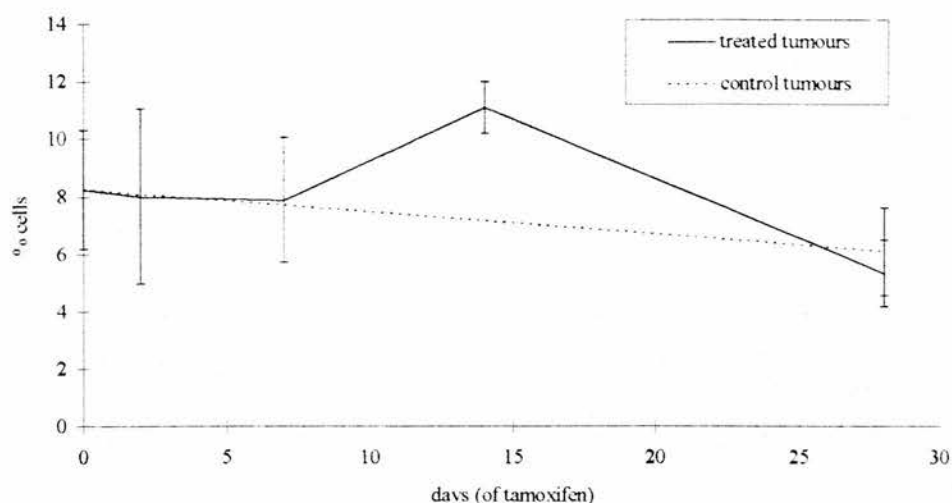


Figure 3.6.1 FACS-estimated apoptosis in tamoxifen treated and control ZR-75-1 xenografts

3.6.2 Cell-cycle analysis

The data from the FACS analysis of the proportions of cells in the different parts of the cell cycle are shown in figure 3.6.2, where the trend can be seen for both S-phase and G2/M to fall with tamoxifen treatment. For G2/M this is significant on day 7 ($W = 114.5$, $p = 0.05$), with a trend on day 28 ($W = 135.5$, $p = 0.09$), and a significant overall trend with time ($z = -1.961$, $p < 0.05$). In contrast for S-phase fraction, this is only significant on day 28 ($W = 143$, $p = 0.03$), but nonetheless the overall trend is significantly downwards ($z = -2.864$, $p < 0.005$). There are no differences between the control tumours on day 0 and the two untreated tumours available for analysis from day 28. Data on the proportions of cells in G1 are not shown, as the figures are derived by subtracting from 100% the combined figures for G2/M and S-phase.

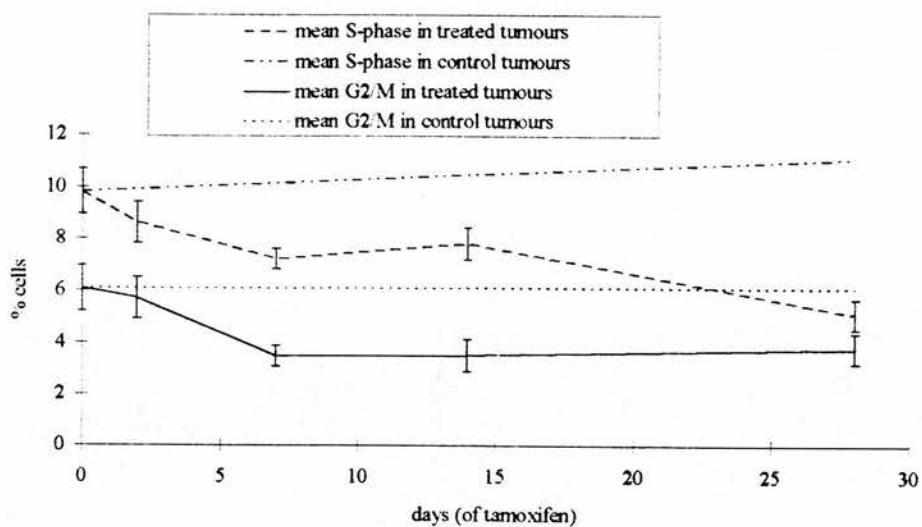


Figure 3.6.2 Cell-cycle analysis in tamoxifen treated and control ZR-75 xenografts

3.7. Correlations

The observations on altered growth, apoptosis, mitosis, MIB-1 expression, necrosis and vascular density are unlikely to have occurred in isolation from each other. However it is more difficult to be certain of how these changes are related. One can propose hypotheses that may on testing reveal direct correlations, but the association does not necessarily imply cause and effect. On the other hand there are 55 possible bi-variate correlations that can be tested, which is further compounded by the treatment/no-treatment variable and the possibility of multiple regression.

Therefore in order to reduce the possibility of a false positive significant association, the relationships to be tested need to be stated in advance, and caution exercised when interpreting relationships that are only significant at the 5% level.

The first set of correlations to be tested relate to the different methods of assessing proliferation : MIB-1 expression, the mitotic index, and the percentage of cells in the G0/G1, S & G2/M phases of the cell cycle. These are summarised in table 3.7.1, where it can be seen that in general the different measures of proliferation are in very good agreement with one another, although there is no correlation between the mitotic index and the proportion of cells in G2/M as measured by flow cytometry.

| Proliferation | MIB-1 | MI | G0/G1 | S-phase | G2/M |
|---------------|------------------|------------------|-------------------|-------------------|-------------------|
| MIB-1 | | 0.652 <0.0001 | -0.508 <0.001 | 0.54 <0.001 | 0.415 <0.01 |
| MI | 0.656 <0.0001 | | -0.374 <0.01 | 0.395 <0.01 | 0.296 ns |
| G0/G1 | -0.45 <0.01 | -0.393 <0.02 | | -0.970 <0.0001 | -0.913 <0.0001 |
| S-phase | 0.504 <0.001 | 0.43 <0.01 | -0.98 <0.0001 | | 0.793 <0.0001 |
| G2/M | 0.342 <0.05 | 0.298 ns | -0.893 <0.0001 | 0.73 <0.001 | |

Table 3.7.1 Correlations between different measure of proliferation for ZR-75-1 xenografts, with the values for the treated tumours given in the lower left part of the table, and the values for all the tumours in the upper right section.

It should be recalled that flow cytometric data were not available for all tumours, and if the analysis is confined to the subset for whom all measures of proliferation are available, the previously noted correlations remain statistically significant, with the exception of the flow cytometric data and the mitotic index for all tumours (although the relations remain significant for the treated tumours).

For the control tumours, the only significant correlations are for MIB-1 expression and the mitotic index. ($r=0.527$, $p < 0.02$). and between the three FACS-derived cell-cycle parameters (all $p < 0.001$). For the non-regressing control tumours, MIB-1 expression also correlates with G0/G1 ($r = -0.854$, $p < 0.01$), S-phase ($r = 0.869$, $p < 0.01$) and G2/M ($r = 0.764$, $p < 0.02$). These results suggest that the measures of proliferation are all, as expected, related. The notable exception is the lack of any correlation between the Mitotic index and the percentages of cells in G2/ M. This could be due to the fact that the flow cytometer cannot distinguish between cells in G2 and M on the basis of their DNA content, and therefore the mitotic index is not measuring the same group of cells as the flow cytometer. The weaker correlations within the control tumours could be a reflection of the smaller numbers. Multiple regression analysis can be done 5 ways, but consistently one finds that the G0/G1 variable drops out, which is not surprising as it is calculated from two others being considered (G2/M and S-phase fraction). Omitting this variable then, for the MI, only the MIB-1 coefficient is significant ($R^2 = 0.26$, $t = 3.29$, $p < 0.01$) ; for MIB-1 both S-phase fraction & MI are significant ($R^2 = 0.40$, respectively $t = 2.67$ & $t = 3.29$; both $p < 0.01$). For S-phase fraction and G2/M, perhaps not surprisingly it is the only other cell-cycle measure which is significant ($R^2 = 0.64$, $t = 7.05$, $p < 0.001$). Thus although the different measures of proliferation are related, they tend to fall into two groups ; FACS measures and histological measures, each of which have tighter correlation within the group than with the other group ; the notable exception is the percentage of cells positive for MIB-1, which is consistent with the property of the MIB-1 antibody to detect proliferating cells in G1, S-phase & G2/M (Cattoretti et al(1992); McCormick et al(1993)). Furthermore these data are consistent with studies in clinical breast cancer which report significant correlations between the level of Ki-67 expression (to which MIB-1 is a highly specific monoclonal antibody (Cattoretti et al(1992))) and both S-phase fraction (Gasparini et al(1994a); Keshgegian et al(1995)) and mitotic index (Weidner et al(1994)).

Similarly the correlations between the measures of cell-death are shown in table 3.7.2. Perhaps surprisingly it appears that the histological measures of apoptosis and necrosis are correlated, whereas the flow cytometric measure of apoptosis does not correlate with either histological assessment. If the analysis is restricted to those tumours for which all three measures are available, then the only significant correlation is between the apoptotic index and necrosis in the treated tumours.

| Cell Death | apoptotic index | necrosis | FACS-TUNEL |
|-----------------|-----------------|----------------|------------|
| apoptotic index | | 0.227 <0.05 | ns |
| necrosis | 0.321 <0.01 | | ns |
| FACS-TUNEL | ns | ns | |

Table 3.7.2 Correlations between measures of cell death in ZR-75-1 xenografts, with the treated tumours represented by the lower, left part of the table, and all tumours in the upper right section.

There is also a significant correlation between the apoptotic index and necrosis in untreated tumours, but in the opposite direction ($r = -0.45$, $p < 0.05$). However multiple regression analysis reveals no significant correlation for the apoptotic index :

$$\text{Apoptotic index} = 0.006 + 0.006 * \text{necrotic proportion} + 0.00006 * \text{FACS-TUNEL}$$

| Predictor | t-ratio | | |
|------------|---------|------|--------------|
| Constant | 6.4 | | |
| FACS-TUNEL | 0.74 | n.s. | |
| necrosis | 1.4 | n.s. | $R^2 = 5 \%$ |

As far as the FACS measure of apoptosis is concerned. the multiple regression analysis suggests that it is only significantly related to the amount of necrotic tissue (as it will tend to make a larger contribution in the following equation) , and not the proportion of cells that are apoptotic morphologically :

$$\text{FACS-TUNEL} = 4.67 + 150 * \text{Apoptotic index} + 15.2 * \text{necrotic proportion}$$

| Predictor | t-ratio | | |
|-----------------|---------|------------|--------------|
| Constant | 2.54 | | |
| apoptotic index | 0.74 | n.s. | |
| necrosis | 2.08 | $p < 0.05$ | $R^2 = 8 \%$ |

However the small R^2 value suggests that even including the necrotic proportion only predicts a small part of the FACS measure of apoptosis.

Finally, there are possible relationships between cell proliferation and cell death, as well as with the vascular supply. Rather than consider all measures of proliferation, the FACS-measures will be discounted, except when also considering another FACS-measure.

| Necrosis | MIB-1 | M.L. (univariate) | M.L. (multivariate) | vasculature |
|-----------------|-----------------|----------------------|------------------------|-------------|
| all tumours | 0.528 <0.001 | 0.582 <0.001 | t = 4.1 < 0.001 | ns |
| treated tumours | 0.481 <0.001 | 0.53 <0.01 | t = 3.1 < 0.01 | ns |
| control tumours | 0.55 <0.05 | 0.46 ns | t = 2.4 < 0.05 | ns |

Table 3.7.3. Correlations between necrotic proportion of the tumour and measures of proliferation and vascular supply.

Table 3.7.3 demonstrates that the necrotic proportion of the tumour is strongly correlated with proliferation, and not with the vascular supply. The observation that this relationship holds in the untreated as well as the treated tumours gives further credence to the observation that necrosis in these xenografts is not affected directly by tamoxifen treatment. A positive relationship between the rates of apoptosis and mitosis are suggested by table 3.7.4, with further confirmation that proliferation and apoptosis are linked by the correlation with MIB-1 expression. The lack of significance in the control tumours could be due to the smaller numbers.

| Apoptosis | MIB-1 (univariate) | MIB-1 (multivariate) | M.L. | vasculature |
|-----------------|-----------------------|-------------------------|-----------------|-------------|
| all tumours | 0.362 <0.01 | t = 3.1 < 0.01 | 0.344 <0.001 | ns |
| treated tumours | 0.486 <0.001 | t = 4.1 < 0.001 | 0.424 <0.001 | ns |
| control tumours | ns | ns | ns | ns |

Table 3.7.4. Correlations between the apoptotic index of the tumour and measures of proliferation and vascular supply.

Table 3.7.5 suggests that the flow cytometric measure of apoptosis has similar correlations to the histological measures of cell death, although weaker, as suggested by the lower p-values and the lack of a significant association with the mitotic index.

| FACS-TUNEL | MIB-1 (univariate) | MIB-1 (multivariate) | M.I. | S-phase | G2/M | vasculature |
|------------------------|-------------------------------|---------------------------------|-------------|----------------|-------------|--------------------|
| all tumours | 0.324 <0.01 | t = 3.3 < 0.01 | ns | ns | ns | ns |
| treated tumours | 0.425 <0.01 | t = 3.9 < 0.001 | ns | 0.395 <0.02 | ns | ns |
| control tumours | ns | ns | ns | ns | ns | ns |

Table 3.7.5. Correlations between the flow cytometric measure of apoptosis of the tumour and measures of proliferation and vascular supply.

Table 3.7.6, together with tables 3.7.3 - 3.7.5, demonstrates that the vascular proportion of the tumour is not in general directly related to any of the other measures in the treated tumours. In contrast, in the small group of untreated tumours, there are apparently contradictory associations with proliferation ; positive with the flow cytometric data and negatively with the mitotic index.

| Vasculature | MIB-1 | M.I. (univariate) | M.I. (multivariate) | S-phase | G2/M |
|------------------------|--------------|------------------------------|--------------------------------|----------------|----------------|
| all tumours | ns | ns | ns | ns | ns |
| treated tumours | ns | ns | ns | ns | ns |
| control tumours | ns | -0.629 <0.05 | t = -2.4 <0.05 | 0.763 <0.05 | 0.727 <0.05 |

Table 3.7.6. Correlations between the vascular proportion of the tumour and measures of proliferation.

3.8 Discussion

It is helpful therefore to summarise the trends noted in the preceding paragraphs, as in table 3.8.1, where comments in parentheses refer to data that are not statistically significant at the 5% level, but could still be meaningful as suggested by other corroborative evidence.

| | control tumours | treated tumours |
|-------------------------|------------------------|------------------------|
| growth | increasing | regression |
| apoptosis | no change | transient increase |
| mitosis | no change (? decrease) | decreased |
| apoptotic/mitotic ratio | no change (? increase) | increase (?sustained) |
| MIB-1 expression | no change (? decrease) | decreased |
| necrosis | no change | decreased |
| vasculature | no change | decrease (? sustained) |
| FACS G2/M | no change | decrease |
| FACS S-phase fraction | no change | decrease |
| FACS-TUNEL | no change | no change |

Table 3.8.1. Summary of changes seen in tamoxifen treated and control ZR-75-1 xenografts over a 28 day period.

For the control tumours the only significant change over time is an increase in the tumour size, although there is a suggestion that the proliferative fraction (as assessed by the mitotic index and MIB-1 expression) falls. Consistent with this is the suggestion of an increase in the apoptotic/mitotic ratio. These changes appear to be largely confined to the 4/10 of the control tumours removed on day 28 that regressed towards the end of the experiment. Thus figures 3.2.1 - 3.5.1 also show the values for the non-regressing control tumours removed on day 28 where, although they appear to differ from the values for the group as a whole, in no case were the differences statistically significant. Hence it may be concluded that the growth rate of all the control tumours was not consistent over time, but this does not appear to be as a consequence of changes in vascular supply (as there were no changes in necrosis or vasculature).

Figure 3.1.1 demonstrates that tamoxifen causes regression of the oestrogen-receptor positive ZR-75-1 breast cancer xenografts, and therefore the differences between the treated and control tumours can be considered to occur at least in association with such regression. As figure 3.2.1 shows, within two days of tamoxifen therapy there was a significant increase in apoptosis, with the mean level rising from 0.7% to 1.05%, which

translates to a 50% increase in cell loss, the same value as that calculated by Brunner for tamoxifen treated MCF-7 xenografts (Brunner et al(1989)). On day 7 the average value is almost identical, but the range is wider, which may account for the lack of statistical significance when comparing with either group of control tumours. However when combining the day 2 & 7 values and/or the control values, the rise in apoptosis becomes significant. (These data are consistent with most (but not all (Kristensen et al(1995b); Szende et al(1990))) prior data on the effect of oestrogen deprivation or antagonism (Frankfurt, (1995); Kyprianou et al(1991b); Perry et al(1995a); Szende et al(1989); Szende et al(1990); Warri et al(1993); Warri et al(1993))). Thereafter the value appears to fall, to around the same value as in the untreated tumours, and this is also consistent with similar studies conducted on the effect of hormone-deprivation of prostate cancer cells (Kyprianou et al(1990); Martikainen et al(1990)), as well as oestrogen-deprivation of hormone-sensitive MCF-7 breast cancer xenografts (Kyprianou et al(1991b)). The specificity of these changes in apoptosis to tamoxifen remains uncertain, as there were no control tumours at these early time points. The values in the day 28 control tumours are similar to the day 0 values, but a proportion of the former regressed towards the end of the experiment.

In contrast to the pattern observed with apoptosis, figure 3.2.2 confirms previous observations that tamoxifen induces a sustained decrease in proliferation (Brunner et al(1989); Butler et al(1988); Danova et al(1993); Kute et al(1985); Osborne et al(1983); Osborne et al(1985); Sarkaria et al(1993); Sutherland et al(1987)), and the fall in MIB-1 expression (figure 3.3.1) corroborates this. Section 3.6 confirms that the methods used to assess proliferation (mitotic index, MIB-1 expression and the proportions of cells in G2/M and S-phase) are in general in agreement with one another. Figures 3.2.2 and 3.6.2 suggest that MIB-1 is less sensitive to the earliest changes in proliferation, as there are no significant differences in this marker until day 21 of treatment, although there is a consistent trend for less proliferation with increased duration of tamoxifen therapy. There is also the suggestion that the proliferative fraction may fall with time in the control tumours, although this is to a large extent confined to the regressing tumours.

The apoptotic:mitotic ratio serves as a measure of the relative balance between proliferation and cell loss. Figure 3.2.3 demonstrates that this rises significantly over the first 7 days of tamoxifen therapy, and then falls, but not to its original level. However the control tumours also have an apparent but non-significant rise in the ratio

over the same period, explained only in part by the regressing tumours. Hence tamoxifen appears to alter the relative balance between cell proliferation and apoptosis. Table 3.7.4 confirms that there is a positive relation between the levels of apoptosis and proliferation in these tumours. The lack of a significant relationship in the control tumours could be due to the suggestion that the apoptotic:mitotic ratio is higher in the regressing day 28 controls. There are no previous reports of the effect of tamoxifen upon this ratio in xenografts.

The above changes in apoptosis occur without any detectable change in the level of expression of either bcl-2 or p53. The latter is not particularly surprising, as p53 is implicated in the induction of apoptosis in response to genetic damage, whereas the expectation is that tamoxifen acts by inhibiting growth, and is therefore likely to act in a p53-independent manner. What is a little more surprising is the lack of any change in the expression of bcl-2. The technique employed, namely immunohistochemistry, may be partly responsible, as it may be insensitive to small, transient changes, particularly if those changes only occur in cells just prior and during apoptosis, as the condensation of the cytoplasm that occurs during apoptosis could conceivably be sufficient to mask any reduction in concentration induced by tamoxifen. However it is more likely that if tamoxifen induces apoptosis by inhibiting bcl-2 expression, it would do so in many (or all) sensitive cells, and thus be visible across the section. Equally it is possible that it is altered expression of another member of the bcl-2 family (Krajewski et al(1995); Oltavi et al(1993); Sumantran et al(1995); Teixeira et al(1995); Wang et al(1995); Yang et al(1995)) that increases the propensity for apoptosis, but the lack of reliable monoclonal antibodies has prevented the testing of this hypothesis.

There is a clear fall in the proportion of each ZR-75-1 tumour that is necrotic during tamoxifen therapy (see figure 3.4.1). In the control tumours there is also the suggestion of a fall, but this mostly due to the lower values in the regressing day 28 control tumours. Section 7 will confirm confirms that the proportion of each treated tumour that is necrotic is highly significantly correlated with all measures of volume or response, consistent with its progressive fall on treatment. Such a relation could therefore explain the apparent fall in the regressing day 28 tumours ; if they are regressing because of oestrogen deprivation, then the data from the tamoxifen treated tumours suggests that they too might manifest less necrosis. These data are however in contradiction with a report of the effect of MCF-7 xenografts treated with a higher dose of tamoxifen (Haran et al(1994)), where the necrotic portion was measured non-

invasively by MRI scanning. Unpublished observation from our own laboratory (Dr. J Keen) suggest that higher doses of tamoxifen are associated with more necrosis, which could therefore be a non-specific effect of the drug. The data in the present study however do not support the notion that any of the increased cell-loss occurring as a result of tamoxifen therapy is due to necrosis.

Figure 3.5.1 shows that tamoxifen therapy is associated with a fall in the vascular proportion of the tumour. This is consistent with previous reports of tamoxifen having an anti-angiogenic action (Gagliardi et al(1993)). It is interesting that there are no correlations between the level of vasculature and the amount of necrosis (see table 3.7.3) - a previous study has suggested that there were changes in the vasculature as well as increased necrosis in tamoxifen treated xenografts (Haran et al(1994)). In the control tumours the data suggest that the more vascular tumours have a higher proliferative fraction, as assessed flow cytometrically, but not with MIB-1 (see table 3.7.6). It should be recalled that only 13/21 control tumours were available for assessment of vasculature and this could result in a false negative correlation. What is more difficult to explain is the inverse relation with mitotic index. Section 3.7 pointed out that the correlations between proliferative markers were weaker in the control tumours, and in particular the mitotic indices were not associated with any flow cytometric parameters in this group of cells. Furthermore the control group of tumours include three differing groups : day 0 untreated tumours, day 28 untreated non-regressing and day 28 regressing tumours. As has already been discussed, there is a trend for the regressing tumours to have less proliferation, and only one of these tumours was available for assessment of vasculature, as opposed to 5/6 non-regressing tumours. In contrast FNAs were only available for two of the day 28 control tumours (one regressing and one non-regressing) so that the correlation between cell cycle parameters and vasculature is really a reflection of the situation in the day 0 tumours only.

4. MDA-MB-231 Xenografts

MDA-MB-231 human breast cancer xenografts were, as explained in the methods, derived from two separate passages (Nos. 2 & 7). There were, with one exception, only minor differences between the results for the tumours from these different passages, but none that alter the overall conclusions : therefore all the results have been combined to simplify the data presented. The exception was that the proliferation in the tumours from the later passage, as assessed by the mitotic index, was consistently and significantly higher than in those from the earlier passage ($W = 140, p < 0.005$), see figure 4.1. This phenomenon has been previously noted (Steel et al(1971)), and although significant between these two passages, does not alter the overall conclusions for this section. As in section 3, the data presented have been grouped according to treatment status and the time point when the tumour was removed. Results and the correlation with tumour growth for individual tumours will be covered subsequently in section 7 for clarity.

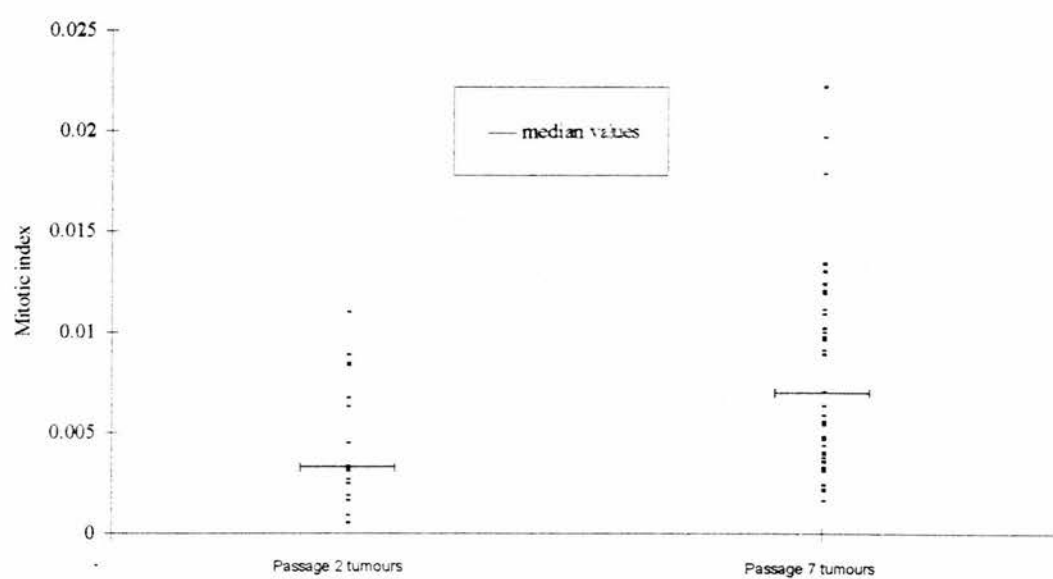


Figure 4.1 Mitotic indices in the MDA-MB-231 tumours from passages 2 & 7

4.1. Growth

The growth curves for the combined data set are shown in figure 4.1.1, where it needs to be pointed out that the data for the day 28 volumes only come from passage 2, and there is a highly significant difference in the volumes of the day 21 control tumours (all from passage 7) as compared with the day 28 control tumours (all from passage 2) ($W = 10, p < 0.025$). This is due to a different growth curve overall for the day 28 control tumours, as shown in figure 4.1.2. Further examination of the growth curves of the tumours from the two passages shows that the treated tumours from the later passage also grew faster, as figure 4.1.3 illustrates. There are however no statistically significant differences between the treated and control tumour volumes at comparable time-points.

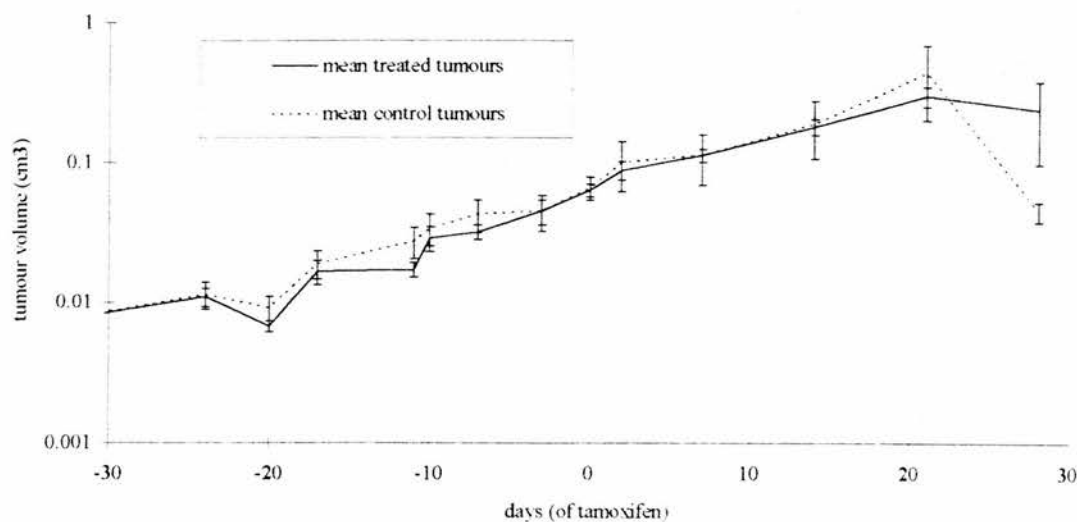


Figure 4.1.1 Growth data for tamoxifen treated and control MDA-MB-231 xenografts

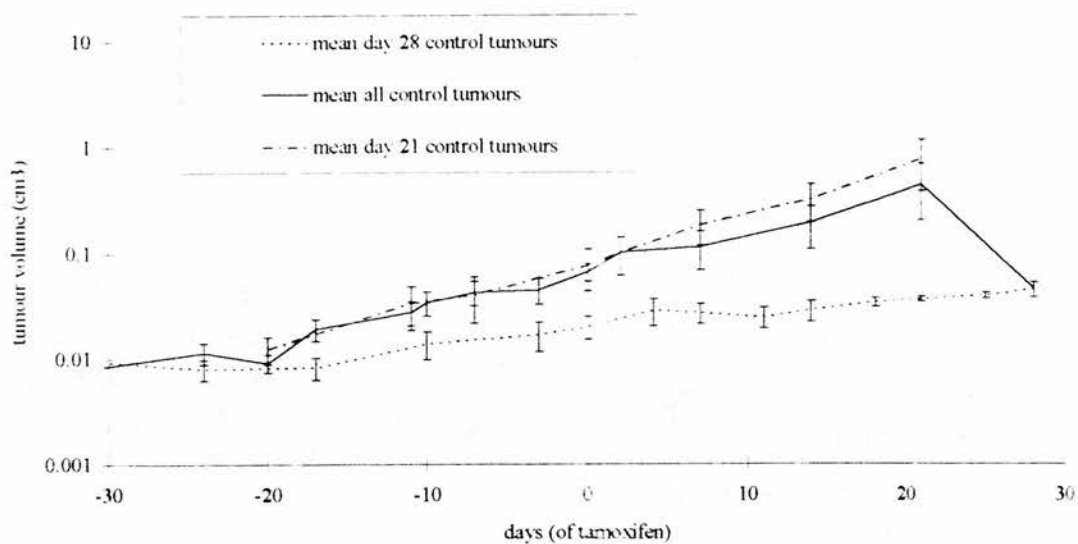


Figure 4.1.2. Growth data for control MDA-MB-231 xenografts

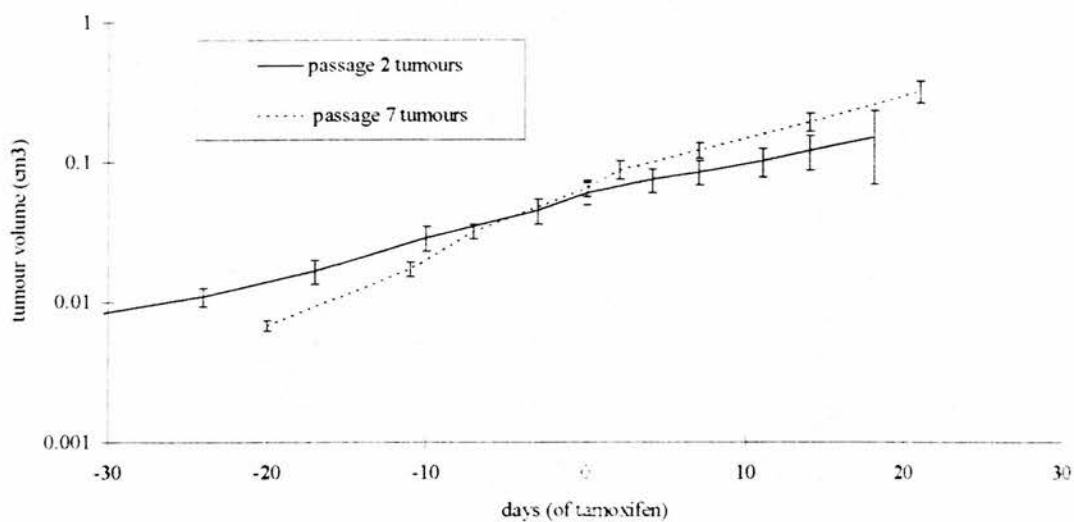


Figure 4.1.3 Growth data for tamoxifen treated MDA-MB-231 xenografts according to passage number

4.2 Apoptosis (& necrosis)

All tumours were examined for mitosis and apoptosis as described in the methods. In general the incidence of apoptotic figures was similar to that found in the ZR-75-1 xenografts. The results are shown in figure 4.2.1, where it can be seen that there is no evidence of a change in apoptosis with tamoxifen treatment, nor, as evidenced by the control tumours, with time. The majority of the MDA-MB-231 tumours had a large central necrotic core, and thus the tissue studied was on the periphery of the tumour. Furthermore the size and consistency of the necrotic core was such that during sectioning and fixation, necrotic material exuded from the core, so that no accurate determination of the relative necrotic proportion of the tumour could be made. Hence no comparative study on the effect of time and/or tamoxifen treatment could be conducted on the MDA-MB-231 tumours.

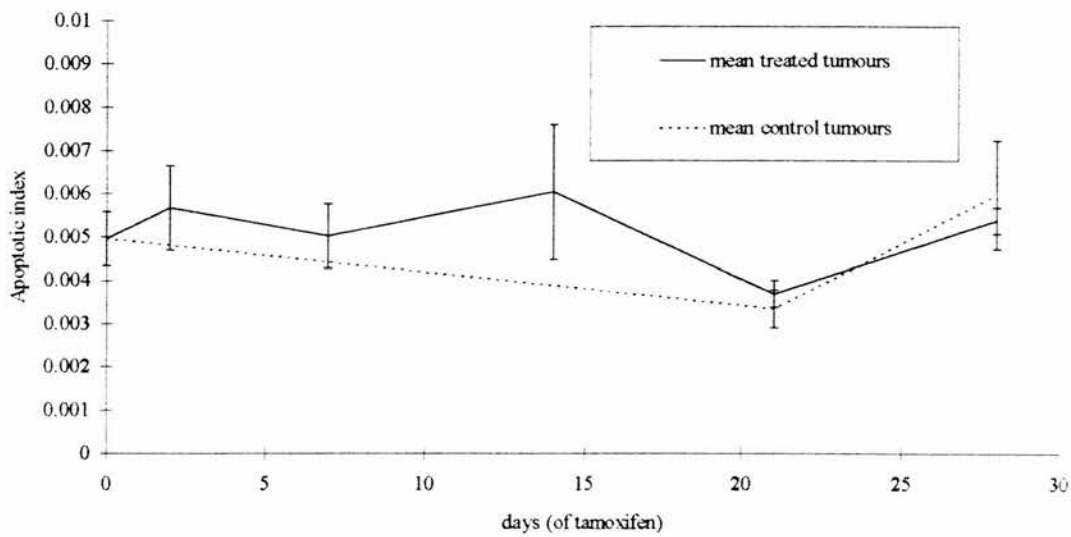


Figure 4.2.1 Apoptotic indices in tamoxifen treated and control MDA-MB-231 xenografts

4.3. Mitosis

Figure 4.3.1 illustrates the mitotic index in the treated and untreated MDA-MB-231 tumours. There is an increase in mitotic index in the treated tumours over time, which is statistically significant ($z = 4.339, p < 0.00005$). This is in part due to the higher proliferation rates seen in the later passage, but not entirely, as comparing all day 0 controls with the day 21 & 28 controls combined there remains a significant difference between them ($W = 83, p < 0.015$). However tamoxifen treatment does not alter the mitotic index, as seen there was no statistical significant difference between the values in the treated and control tumours removed on either day 21 or day 28.

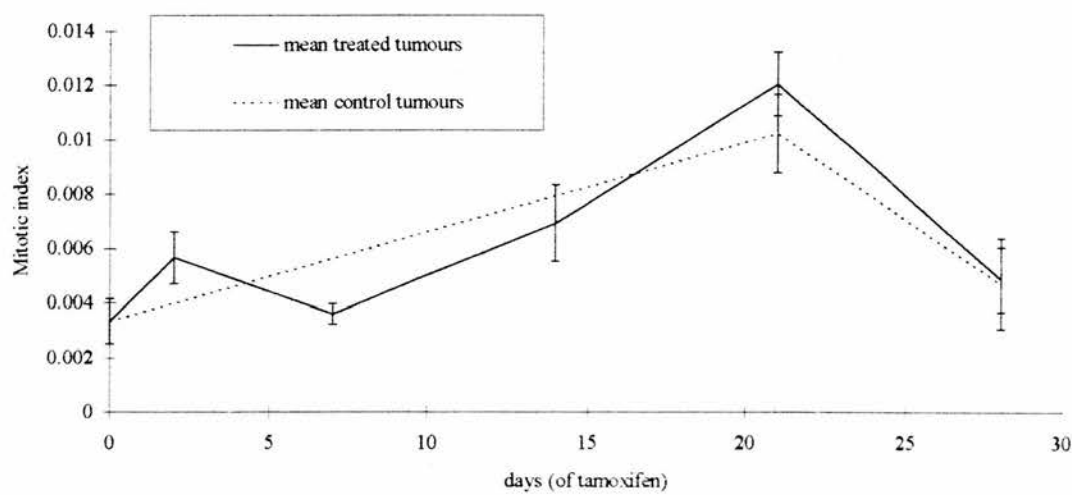


Figure 4.3.1 Mitotic indices in tamoxifen treated and control MDA-MB-231 xenografts

4.4 Apoptotic:Mitotic ratio

The changes in apoptotic:mitotic ratio with time are shown in figure 4.4.1. There are no significant differences between the time points, except for the tumours removed on days 21 & 28, which as previously noted are from different passages with different rates of proliferation. The observed difference in apoptotic:mitotic ratio is statistically significant both for the treated tumours ($p < 0.005$ by t-test) as well as the control tumours ($p = 0.016$ by t-test). Similarly when all treated tumours are compared between the two passages the later passage again has a lower ratio ($p < 0.005$ by t-test). These results are consistent with the previously observed higher proliferation in the later passage. However at any one time point there are no significant differences between the values for the treated and untreated tumours, confirming that tamoxifen does not alter the ratio in the MDA-MB-231 tumours.

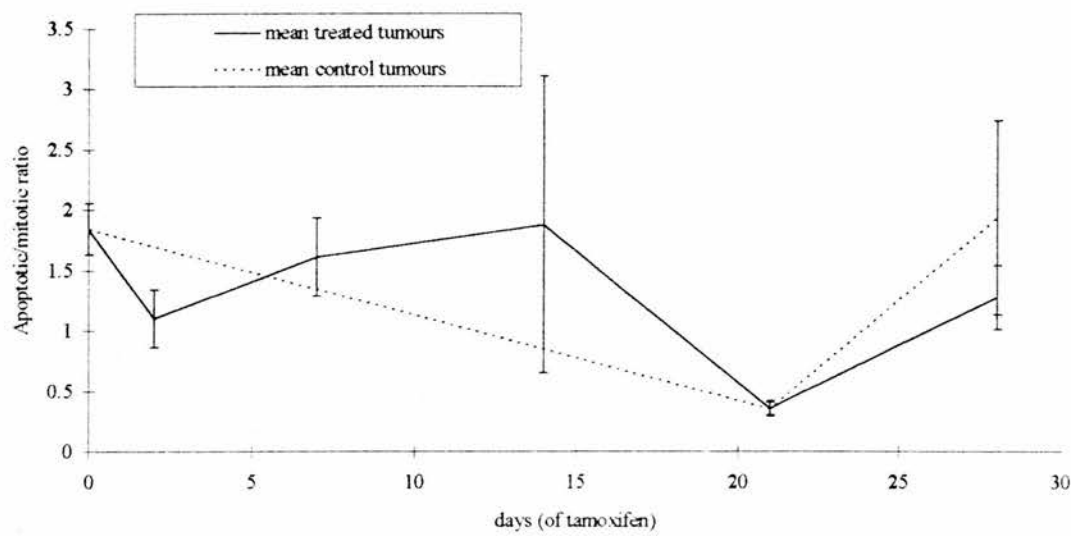


Figure 4.4.1 Apoptotic/mitotic ratio in tamoxifen treated and control MDA-MB-231 xenografts

4.5 Vascular density

As with the ZR-75-1 xenografts (see section 3.5), some MDA-MB-231 tumours were too small to be bisected prior to fixation in paraformaldehyde, or in the case of some of the tumours from the later time points had large necrotic cores and thus were difficult to bisect. Thus table 4.5.1 gives the numbers of tumours available for cryostat sections, where it can be seen that there were representative samples from most time points, the notable exception being the day 28 untreated tumours from passage 2.

| day 0 | day 2 | day 7 | day 14 | day 21 | day 28 | day 21 control | day 28 control |
|-------|-------|-------|--------|--------|--------|-------------------|-------------------|
| 3/11 | 4/6 | 7/10 | 3/8 | 2/16 | 2/4 | 2/5 | 0/4 |

Table 4.5.1 Numbers of MDA-MB-231 xenografts with residual frozen material for cryostat sectioning.

The mean vascular proportion of the tumours is shown in figure 4.5.1, where it can be seen that there is no difference in vasculature at different time points, and that tamoxifen treatment had no effect on the vascular proportion of the MDA-MB-231 tumours.

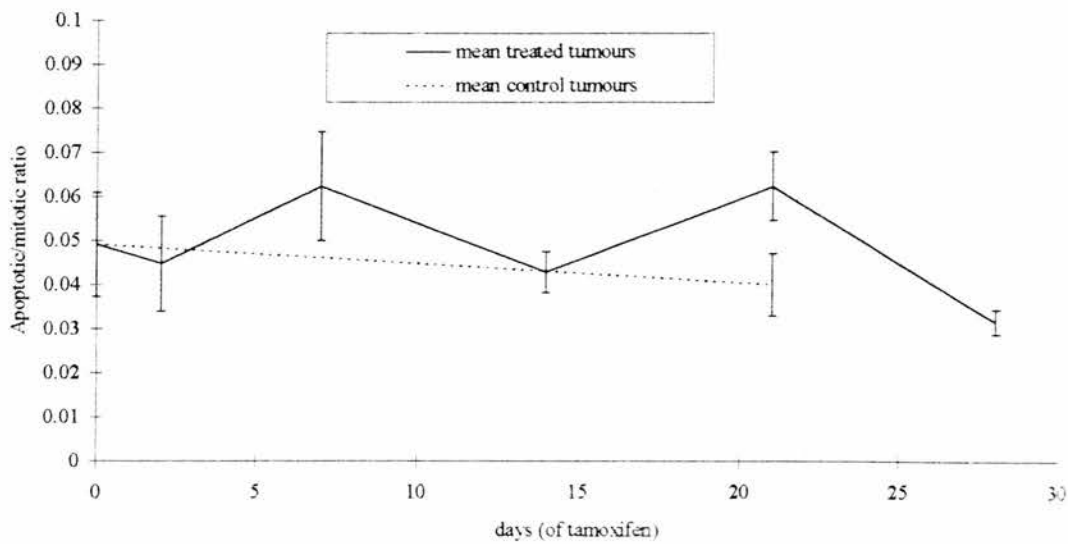


Figure 4.5.1 Vascular proportion in tamoxifen treated and control MDA-MB-231 xenografts

4.6 FACS data

As for the ZR-75-1 tumours, fine needle aspirates were taken to correlate FACScan analysis of apoptosis and proliferation with histological assessments. Not all the FNAs taken were of sufficient quality to permit analysis and table 4.6.1 summarises the available data, in which it can be seen that data are available from all time points.

| | day 0 | day 2 treated | day 7 treated | day 14 treated | day 21 treated | day 21 control | day 28 treated | day 28 control |
|-------------------|-------|------------------|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| cell cycle | 5 | 5 | 7 | 7 | 11 | 5 | 3 | 2 |
| apoptosis | 6 | 5 | 9 | 8 | 11 | 5 | 4 | 4 |
| No. of tumours | 11 | 6 | 10 | 10 | 16 | 5 | 4 | 4 |

Table 4.6.1 Numbers of tumours with available data for FACS analysis of apoptosis and cell cycle.

The data for FACS measures of apoptosis are seen in figure 4.6.1, and those for cell cycle analysis are shown together in figure 4.6.2, with the proportion of cells in G1 not included as this figure is derived from the other two parts of the cell cycle. There are no differences between the values of FACS apoptosis, G2/M or S-phase fraction for treated and control tumours at any time-point. However, when considering the combined treated and control tumours from the final time-point (i.e. day 21 for passage 7 against day 28 for passage 2), significant differences were found in all measures ($W = 67$, $p < 0.05$ for FACS apoptosis, $W = 25$, $p < 0.02$ for G2/M and $W = 151$, $p < 0.025$ for S-phase).

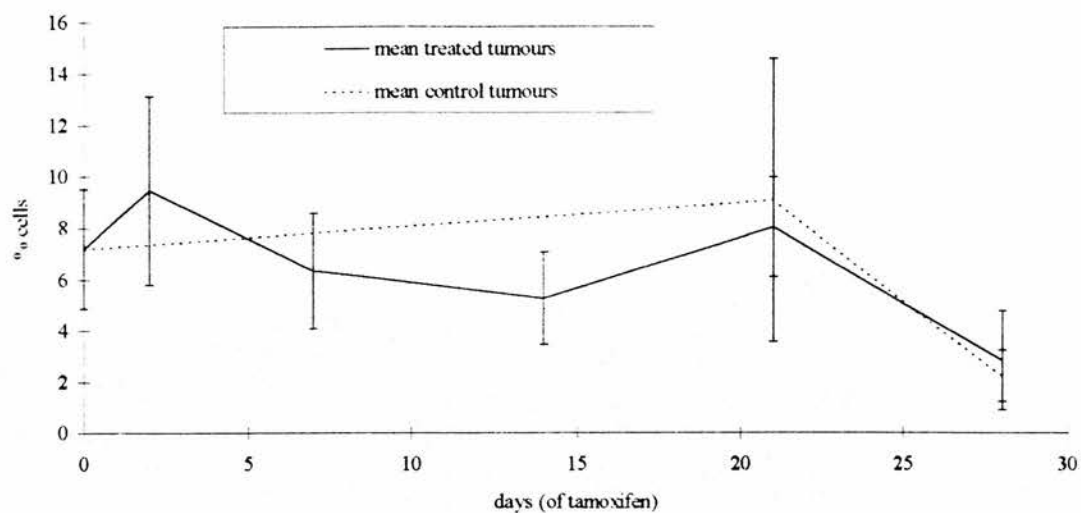


Figure 4.6.1 Percentage of cells in Apoptosis as estimated by FACS for tamoxifen treated and control MDA-MB-231 xenografts

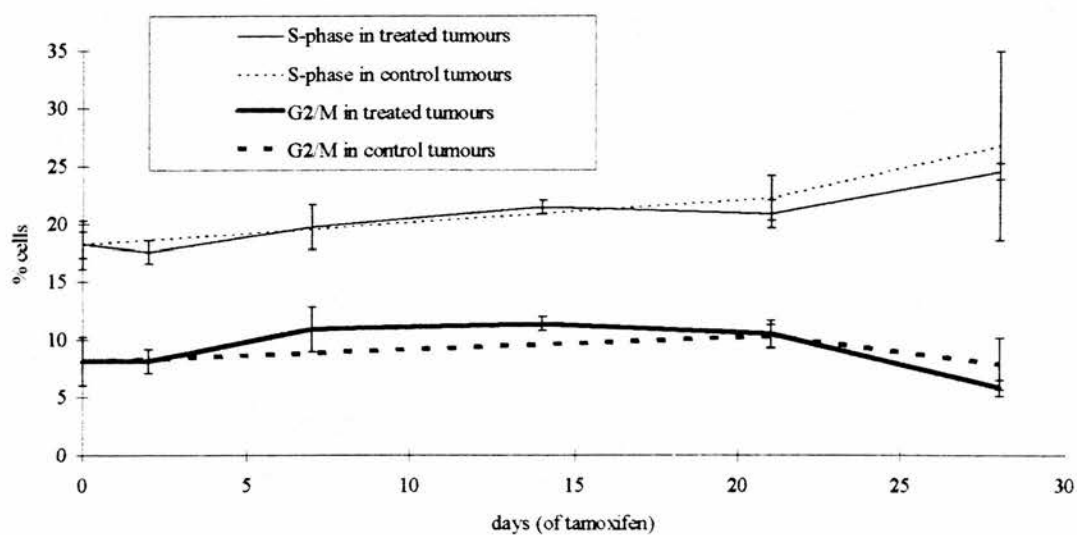


Figure 4.6.2 FACS estimated S-phase and G2/M fraction for tamoxifen treated and control MDA-MB-231 xenografts

4.7 Correlations

Similar analyses as were undertaken in section 3.7 for the ZR-75-1 xenografts, looking at possible correlations between measures of proliferation and cell death can be undertaken for the MDA-MB-231 tumours. However they are perhaps surprisingly almost all negative. The exceptions to this is that for tumours from passage 2 there is a significant correlation between the mitotic index and the S-phase fraction ($r = 0.784$, $p < 0.02$). It should be recalled that there was a significant difference in the proliferation rates for the two passages, and overall a smaller number of tumours in this experiment as compared to the ZR-75-1 experiment. It is possible therefore that false negative results are seen because of this, but in particular the lack of any correlation between the histological and flow cytometric measures of apoptosis should be noted.

4.8 Discussion

The data presented for the MDA-MB-231 xenografts show that tamoxifen treatment has had no significant effect on tumour growth, proliferation or apoptosis. The apoptotic:mitotic ratio, a measure of the overall balance between cell proliferation and death also remains unaltered by tamoxifen therapy. Finally the vascular proportion of the tumour does not change over time, or with tamoxifen treatment. The data presented however do suffer from two potential problems. The first, is that no control tumours were excised at the intermediate time points, and as with the ZR-75-1 xenografts, the values for apoptosis & mitosis in the control tumours excised at the end of the experiment are not unequivocally the same as those for the control tumours from day 0. This may be due to the control tumours which were left to grow unperturbed during the experiment grew at very different rates according to their passage number (see figure 4.1.2) and had significantly different proliferation rates and apoptotic:mitotic ratios. This difference in proliferation rate and growth was a feature of all the tumours. Thus the conclusion that tamoxifen does not alter growth, tumour vasculature, proliferation or apoptosis in these tumours still holds.

It is pertinent to comment on the lack of any pro-apoptotic effect of tamoxifen in these oestrogen-receptor negative xenografts. The data would initially appear to be at odds with a study in which MDA-MB-231 breast cancer cells were cultured and treated *in vitro* with tamoxifen (Kang et al(1996)). It was reported that a $1\mu\text{M}$ concentration of

tamoxifen increased *c-myc* transcription and apoptosis, as detected by inter-nucleosomal DNA cleavage. Furthermore these effects were antagonised by a *c-myc* antisense oligomer. However over the 72 hours that these changes occurred, there was a five-fold reduction in cell number, and sufficient cytotoxicity for DNA laddering to be detectable, whereas it had not been in the control tumours. The current study confirms the existence of apoptotic cells in untreated xenografts, and suggests therefore that the level of apoptosis observed by Perry *et al* (Kang *et al* (1996)) in response to the induction of *c-myc* was several-fold higher, beyond those reported here in xenografts or that likely to occur in clinical tumours. It has been previously reported that *c-myc* overexpression induces significant apoptosis in the presence of growth-factor deprivation (Arends *et al* (1993)). Hence, whatever the mechanism for the *c-myc* induction on exposure to tamoxifen (in an unspecified solvent), the changes seen are consistent with the known effects of *c-myc*, rather than that of tamoxifen. Furthermore 10 μ M tamoxifen in that study was associated with reduced *c-myc* expression, whereas other studies from the same group (Perry *et al* (1995a)) and others (Bardon *et al* (1987)) have suggested that higher levels of tamoxifen induce apoptosis. Finally, a study (using similar techniques) of the effect of oestrogen-withdrawal or treatment with the pure anti-oestrogenic compound ICI 182,780 on the hormone-sensitive cell MCF-7 cell line (Wilson *et al* (1995)) observed decreased cell-number and the presence of DNA fragments, but little evidence for increased apoptosis as assessed by morphology.

5 Mice bearing synchronously grown MDA-MB-231 and ZR-75-1 xenografts

In view of the lack of control tumour material that was removed at the time points when the first changes in apoptosis and mitosis were seen in the ZR-75-1 xenografts, a further experiment was conducted, in which mice bore one ZR-75-1 xenograft and one MDA-MB-231 xenograft, and both treated and untreated tumours were excised on days 2 & 7 (see table 3).

5.1. Growth data

The growth curves of the ZR-75-1 tumours are shown in figure 5.1.1 and those for the MDA-MB-231 tumours in figure 5.1.2. The volumes of tumours excised at each time point are not significantly different between the treated and control groups for both tumour types, although the day 7 ZR-75-1 tumours are significantly smaller than those removed on day 0 ($p < 0.05$).

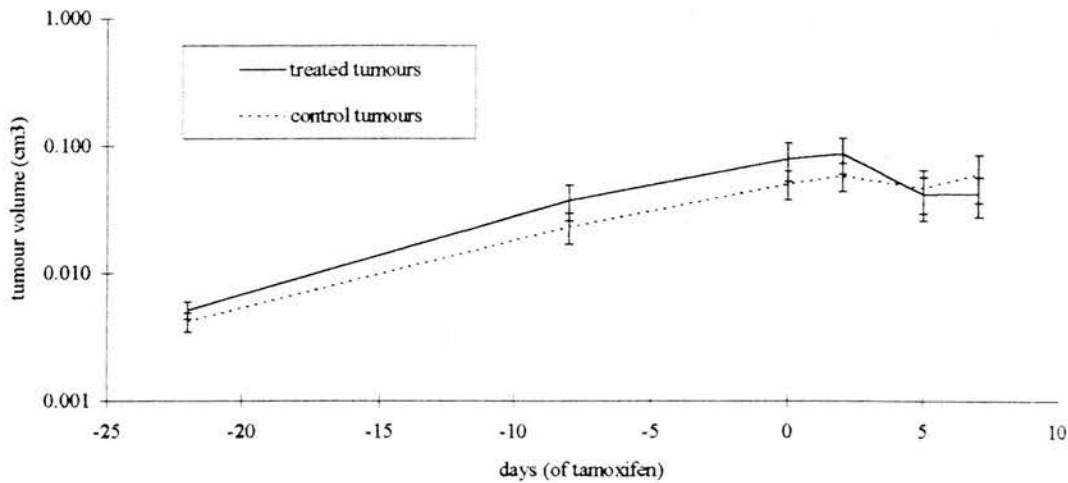


Figure 5.1.1 Growth of tamoxifen treated and control ZR-75-1 xenografts

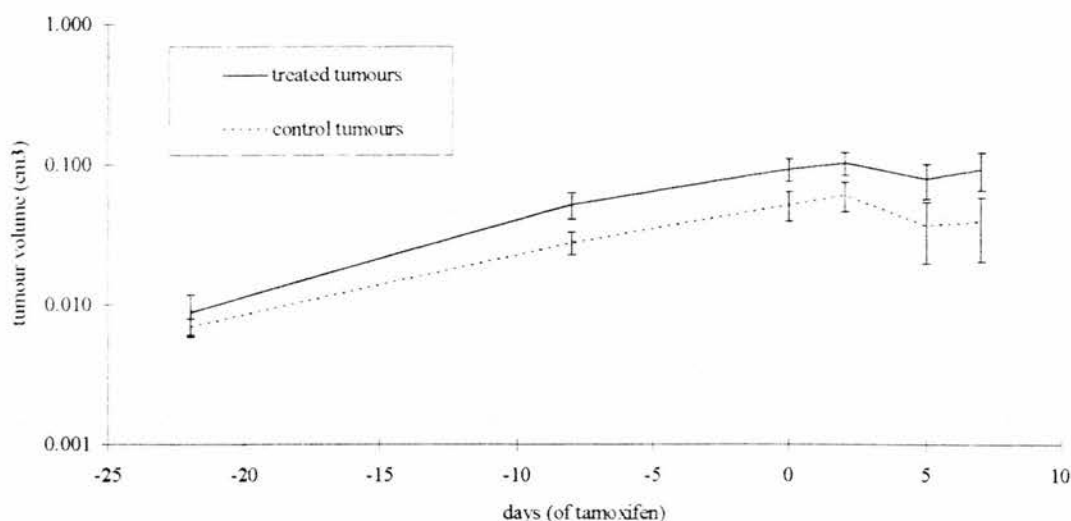


Figure 5.1.2 Growth of tamoxifen treated and control MDA-MB-231 xenografts

There were no significant differences in growth for any group of tumours, with no evidence for tamoxifen induced regression of either ZR-75-1 or MDA-MB-231 xenografts. This is consistent with the data from the previous studies (see sections 3.1 & 4.1), with no significant change in the volume of the tamoxifen treated ZR-75-1 tumour occurring before day 14, beyond the period studied in this experiment.

5.2. Apoptosis, mitosis and the Apoptotic:Mitotic ratio

Apoptosis, mitosis and the ratio of the apoptotic : mitotic indices were determined as previously. The data for Apoptosis and Mitosis is shown graphically in figures 5.2.1 - 5.2.4., where there appears to be a rise in apoptosis in the treated ZR-75-1 but not the treated MDA-MB-231 xenografts, nor in the untreated xenografts of either ZR-75-1 or MDA-MB-231 cells. However statistically there is only a trend towards significance in the ZR-75-1 xenografts on day 2 ($W = 18$, $p = 0.06$). In contrast the mitotic index appears to fall in the treated ZR-75-1 xenografts, but not the untreated controls. This is statistically significant on day 2, where the control tumours have higher mitotic rates than the 2 treated tumours ($W = 38$, $p = 0.04$). The apparent falls in mitotic index in the MDA-MB-231 tumours are not statistically significant. The same pattern as was apparent in the earlier experiments is seen - no difference between treated and control MDA-MB-231 tumours, but a trend for apoptosis to rise and mitosis to fall in the

treated ZR-75-1 tumours. Considering the hypothesis that there should be no differences in the control tumours irrespective of the day of removal, one can compare the treated and untreated tumours by combining the results from days 2 & 7; this increases the number of tumours for the statistical comparisons and thus reduces the possibility of a type II error (a false negative). If this is done, then there is a highly significant difference in apoptosis between the treated and control ZR-75-1 tumours ($W = 61, p < 0.005$), as well as mitosis ($W = 111, p < 0.05$). In contrast there are no such difference for the MDA-MB-231 tumours ($p > 0.2$ in both cases).

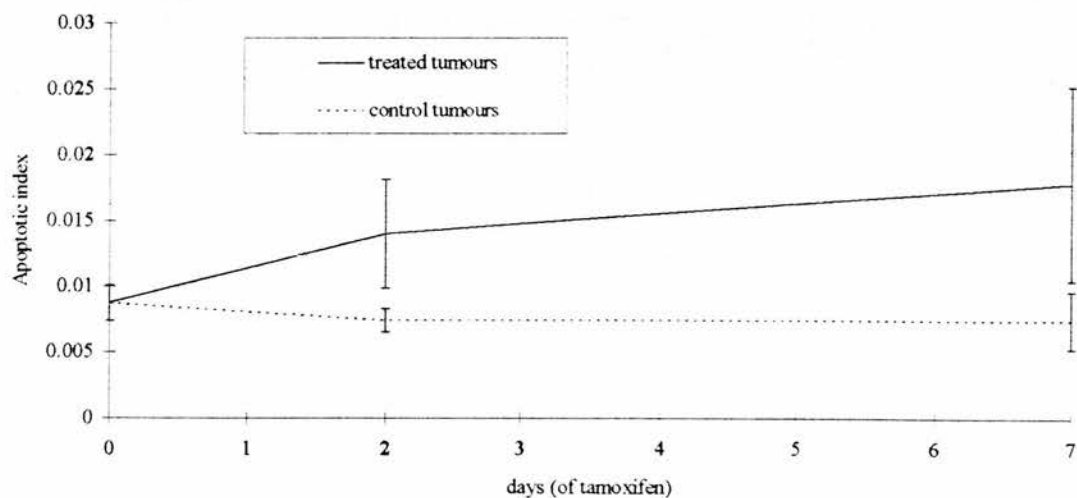


Figure 5.2.1 Apoptotic index in tamoxifen treated and control ZR-75-1 xenografts

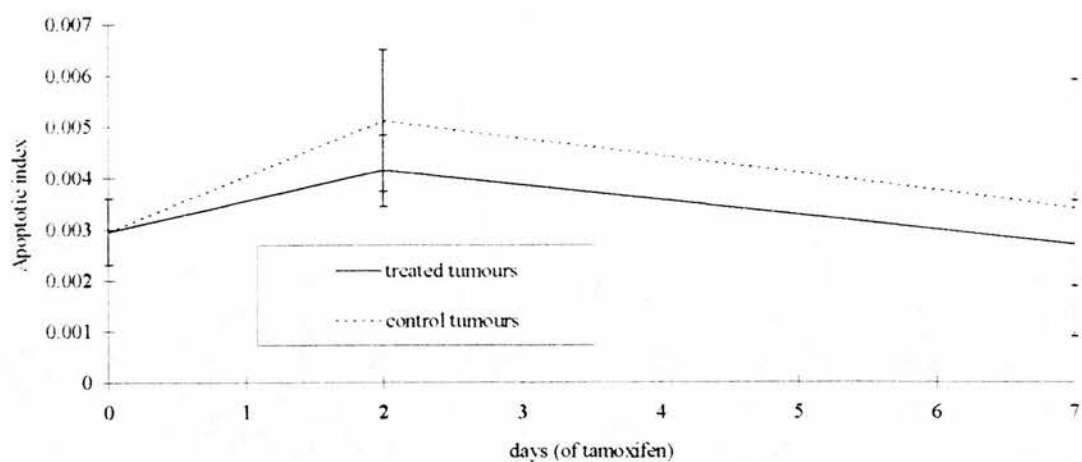


Figure 5.2.2 Apoptotic index in tamoxifen treated and control MDA-MB-231 xenografts

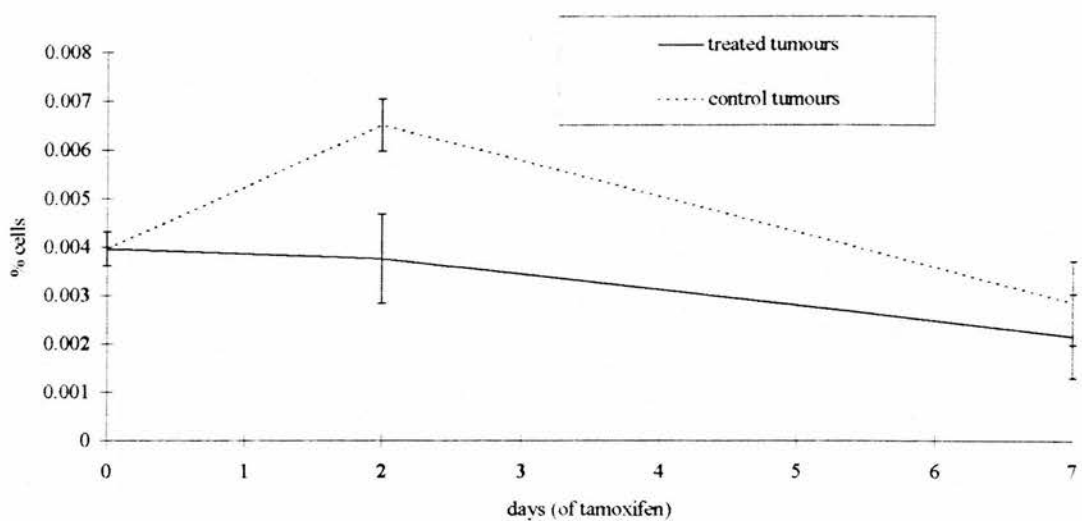


Figure 5.2.3 Mitotic index in tamoxifen treated and control ZR-75-1 xenografts

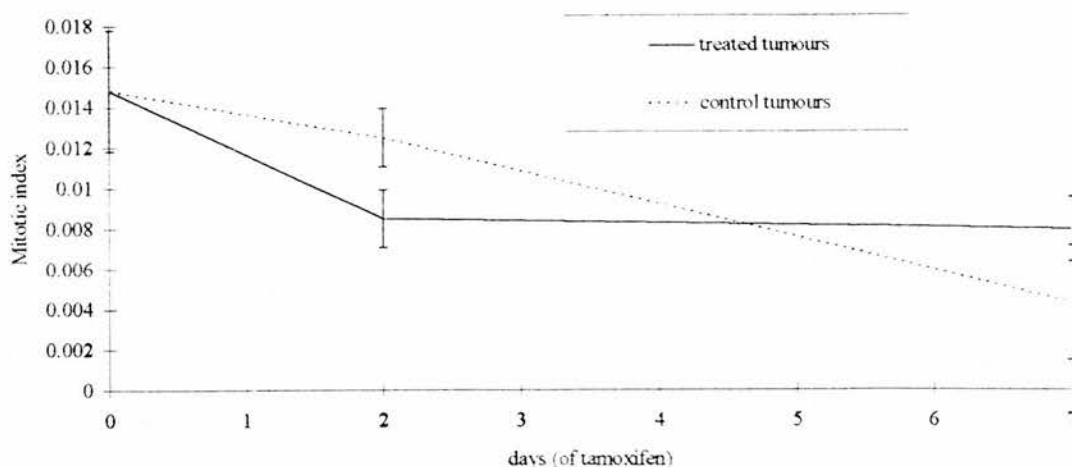


Figure 5.2.4 Mitotic index in tamoxifen treated and control MDA-MB-231 xenografts

When the natural logarithm of the apoptotic:mitotic ratio is considered, significant differences are found. For the MDA-MB-231 tumours, there are no statistically significant differences between treated and untreated tumours (see figure 5.2.6), but for the ZR-75-1, the ratio is significantly higher on day 2 ($p < 0.05$, t-test), although not on day 7 (see figure 5.2.5). However the graph suggests that the ratio continues to increase on day 7, with a trend towards significance ($p = 0.08$), in contrast to the untreated ZR-75-1 and treated MDA-MB-231 tumours ($p > 0.4$ in both cases). Furthermore when considering the combined treated and control tumours from days 2 & 7, the apoptotic:mitotic ratio is significantly higher for the ZR-75-1 tumours ($p = 0.02$) but not for the MDA-MB-231 tumours ($p > 0.5$).

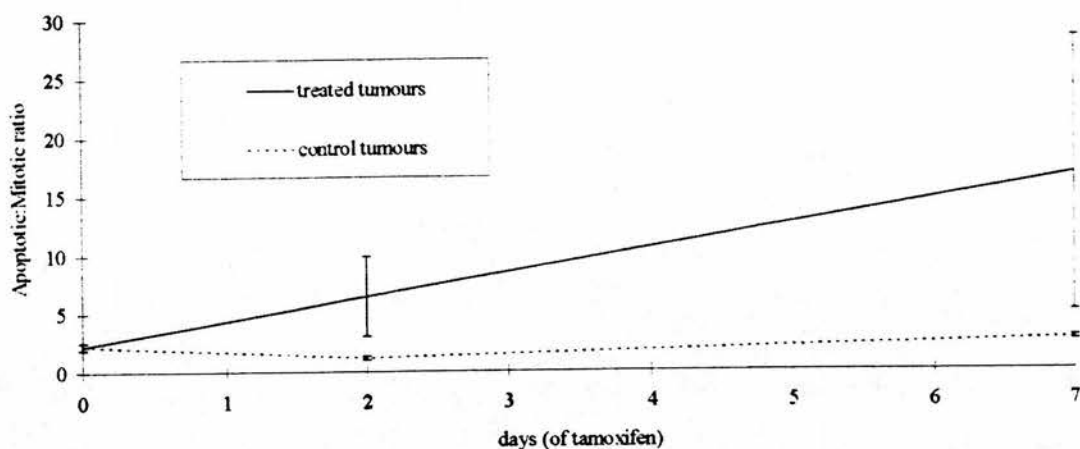


Figure 5.2.5 Apoptotic:Mitotic ratio in tamoxifen treated and control ZR-75-1 xenografts

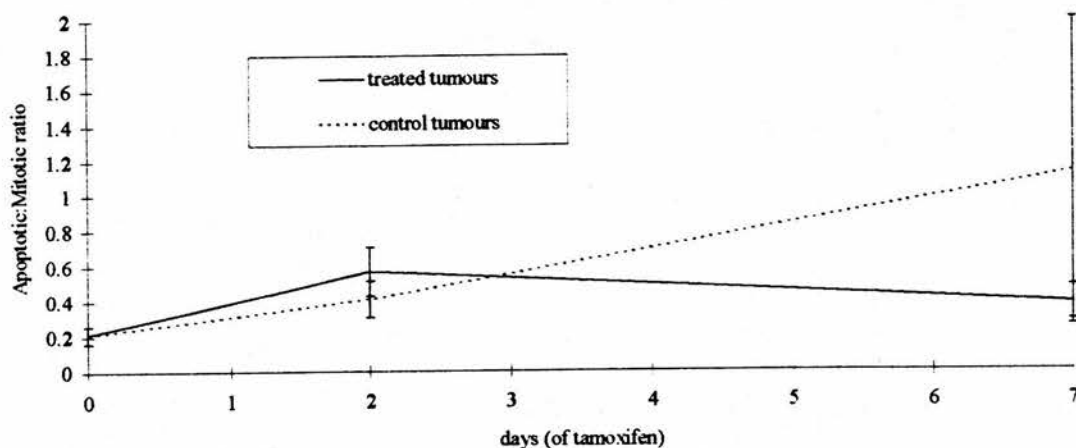


Figure 5.2.6 Apoptotic:Mitotic ratio in tamoxifen treated and control MDA-MB-231 xenografts

5.3 Discussion

This experiment was conducted to confirm that the changes in apoptosis, mitosis and their ratio, observed in the tamoxifen treated ZR-75-1 xenografts, were specifically caused by tamoxifen, the prior experiment being limited by the non-availability of untreated tumour material removed on the days when the changes were maximal, namely after 2 and 7 days' therapy. The pattern seen in this repeat experiment is entirely consistent with the conclusion of the earlier work, namely that tamoxifen induces an increase in apoptosis and a fall in the mitosis, which results in an increase in the apoptotic:mitotic ratio, and that these effects are seen within the first week of therapy only in the oestrogen-sensitive ZR-75-1 breast cancer xenografts. The lack of significant differences at all the individual time points do not detract from these conclusions, as there are no significant changes with time in the untreated tumours, and combining the data from the treated tumours excised on days 2 and 7 confirms that tamoxifen therapy induces a significant increase in apoptosis and reduction in mitosis.

6. Sequential Fine Needle Aspirates of ZR-75-1 and MDA-MB-231 Xenografts

The final experiment involved sequential aspiration of both treated and untreated tumours, in order to determine more precisely the timing of changes in proliferation and apoptosis. The tumours used were ZR-75-1 and MDA-MB-231 xenografts grown from the same passage as the last experiment, with 12 mice bearing a single tumour of each type (a total of 24 animals). FNAs were taken and analysed as described as in the methods, with the exception that the tumours were only removed on day 21.

FNAs were performed on all tumours starting on day 0, before the insertion of the tamoxifen pellets in the treated animals, and again on days 2,5,7,9,12,14, & 21. However one animal died on day 5 (bearing a tamoxifen treated MDA-MB-231 tumour).

6.1 Growth

The growth curves for the ZR-75-1 and MDA-MB-231 tumours are shown in figure 6.1.1 & 6.1.2 respectively. It can be seen that the only tumours to regress are the treated ZR-75-1 tumours, consistent with prior results, although the volumes are only significantly different from the control ZR-75-1 tumours on days 16 ($p < 0.05$), 19 & 21 ($p < 0.01$). There are no significant differences between the volumes of the treated and untreated MDA-MB-231 tumours at any time point.

6.2 FACS data

The design of this experiment was intended to identify the time points at which significant differences in apoptosis and proliferation were to be found between treated and control ZR-75-1 tumours. However not all the FNAs were of sufficient quality to be assessable, and in the case of the treated tumours the proportion of tumour cells in the aspirates fell, such that at day 9 and beyond, few were interpretable, both with regards to the cell cycle soft-ware which has to distinguish between the cells of mouse and human origins, as well as the fluoro-TUNEL technique. The numbers that were available are detailed in table 6.2.1, where it can be seen that the majority of FNAs were of sufficient quality to give dependable data, particularly at the early time points.

| day | Control | | | | Treated | | | |
|-----|------------|------------|------------|------------|------------|------------|------------|------------|
| | ZR-75-1 | | MDA-MB-231 | | ZR-75-1 | | MDA-MB-231 | |
| | Cell cycle | FACS TUNEL | Cell cycle | FACS TUNEL | Cell cycle | FACS TUNEL | Cell cycle | FACS TUNEL |
| 0 | 6 | 6 | 6 | 6 | 5 | 5 | 4 | 4 |
| 2 | 6 | 6 | 6 | 6 | 6 | 6 | 5 | 5 |
| 5 | 6 | 6 | 6 | 6 | 5 | 5 | 5 | 5 |
| 7 | 5 | 5 | 6 | 6 | 5 | 5 | 4 | 4 |
| 9 | 5 | 5 | 6 | 6 | 3 | 3 | 3 | 3 |
| 12 | 6 | 5 | 6 | 6 | 4 | 3 | 4 | 4 |
| 14 | 4 | 4 | 6 | 6 | 3 | 3 | 4 | 4 |
| 21 | 6 | 5 | 6 | 6 | 2 | 1 | 4 | 4 |

Table 6.1.1 Numbers of FNAs from ZR-75-1 and MDA-MB-231 xenografts that were assessable for cell cycle parameters and apoptosis using the FACScan (maximum no. available at any time point was 6).

6.2.1 S-phase & G2/M

There were differences between treated and control ZR-75-1 tumours in the proportions of cells in S-phase and G2/M after day 0, as can be seen in figure 6.2.1. For the treated tumours, the percentage of cells in G2/M was significantly lower on days 2 and 5 ($p < 0.02$), and 7 ($p < 0.01$). On days 9 and 12 there were only 3 treated tumours assessable, with a trend towards significantly ($p < 0.1$) lower values. Beyond that time point there were no significant differences, possibly due to the even smaller number of tumours available for assessment.

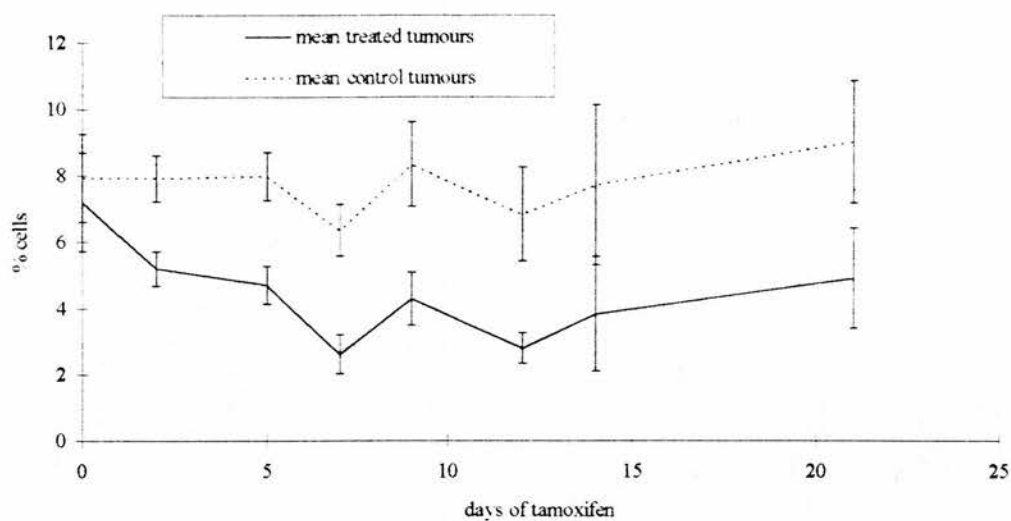


Figure 6.2.1 G2/M in sequential FNAs from tamoxifen treated and control ZR-75-1 xenografts

For the S-phase fraction there is a similar trend towards a lower percentage of cells in S-phase in the treated ZR-75-1 tumours, as shown in figure 6.2.2. However this is only significant on days 7, 9, and 12 ($p < 0.05$), with an apparent rise on days 14 and 21. Although this could be due to the smaller number of available FNAs at these later time points, there is a non-significant rise in the proportion for these same tumours as compared with their values at the earlier time points.

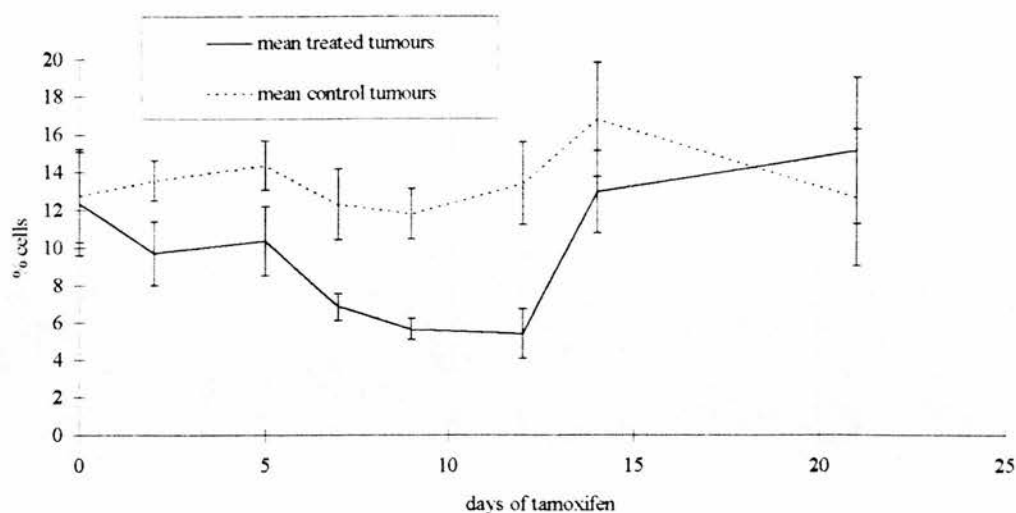


Figure 6.2.2 S-phase in sequential FNAs from tamoxifen treated and control ZR-75-1 xenografts

In contrast there were no differences over time for the S-phase fraction or G2/M population in the MDA-MB-231 tumours, irrespective of whether or not they had been treated with tamoxifen, as is evident from figures 6.2.3 & 6.2.4.

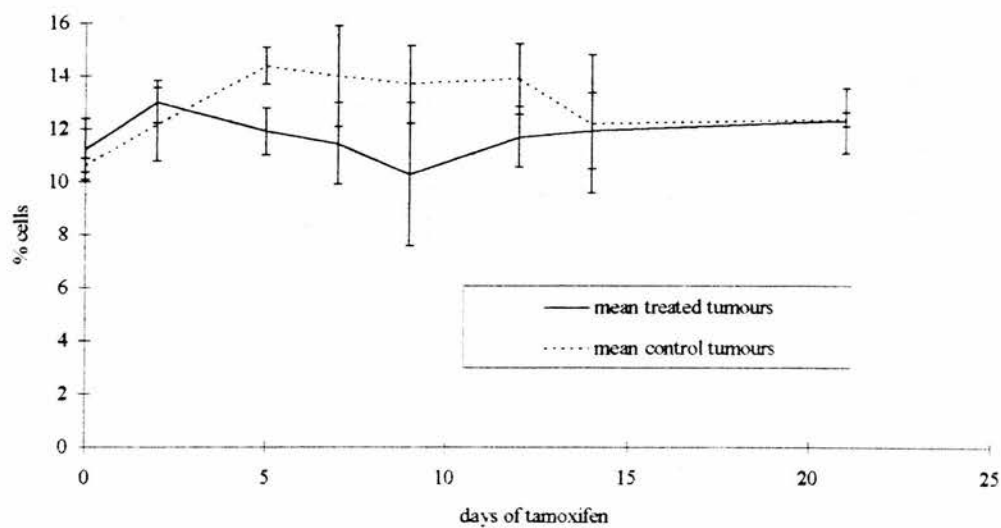


Figure 6.2.3 G2/M in sequential FNAs from tamoxifen treated and control MDA-MB-231 xenografts

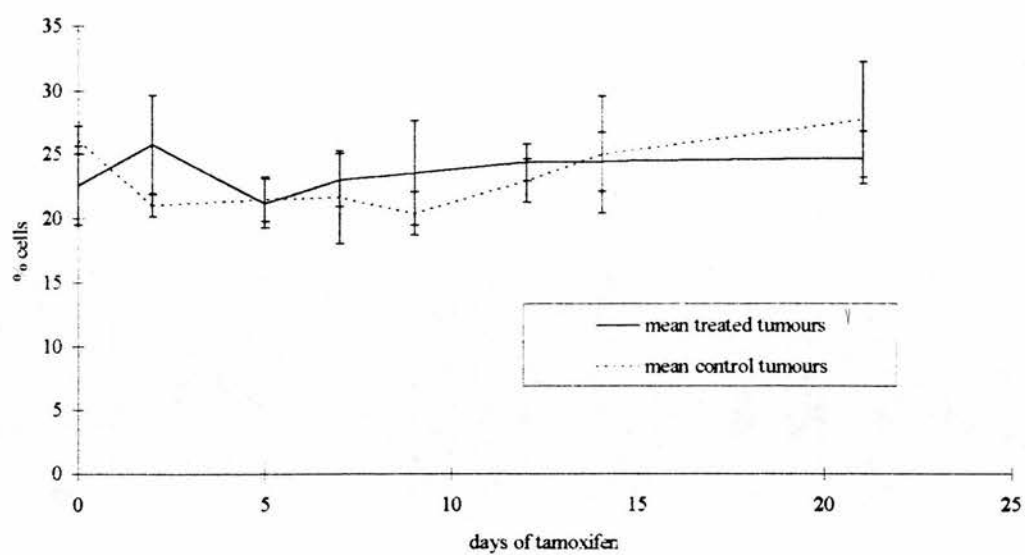


Figure 6.2.4 S-phase in sequential FNAs from tamoxifen treated and control MDA-MB-231 xenografts

6.2.2 Apoptosis and Apoptosis:G2/M ratio

Figure 6.2.5 illustrates the proportions of cells in apoptosis for the treated and control ZR-75-1 xenografts, as measured by FACS-TUNEL. However it can be seen that there is an initial anomaly - the day 0 values for FACS-TUNEL are larger in the treated tumours as compared with the control ($W = 25$, $p=0.06$), at a time just before any tamoxifen has been administered. Therefore the graph includes the two series of tumours with and without all the day 0 values combined. Although there are no significant differences between the values at any individual time-point, if the values on days 2 and 5 (and or 7) are combined, then the treated values are higher ($W = 105$, $p < 0.02$) than the control values. If all the day 0 tumours are combined, there are still no significant differences between the treated and day 0 values, but the control values are significantly less on days 5 ($W = 32$, $p < 0.05$) and 12 ($W = 21$, $p < 0.02$).

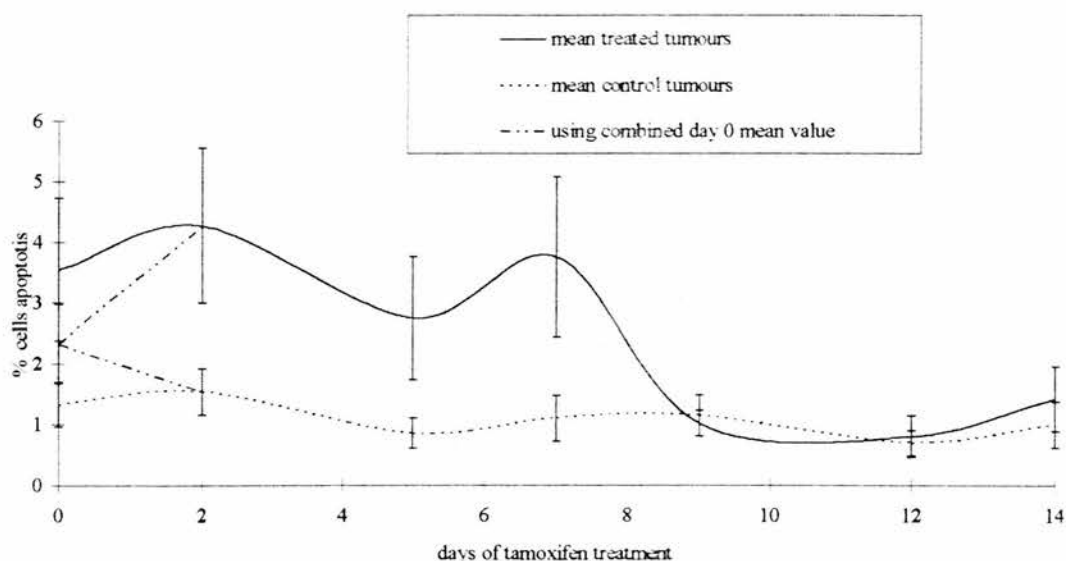


Figure 6.2.5 Apoptosis in sequential FNAs from tamoxifen treated and control ZR-75-1 xenografts

As mentioned in the methods (see page 26), one possible way around the potential problem of identifying DNA fragments in necrotic cells in the aspirates (and thus counting them as apoptotic) is to pre-treat the samples with DNAase. This was performed for the ZR-75-1 tumours with sufficient remaining material (5 treated and 4 control tumours) after a further period of storage which therefore introduced a freeze-thaw cycle which could reduce the viability of the samples. The data are shown in figure 6.2.6, where it can be seen that the initial difference between the day 0 samples

has been reduced, but so apparently have the subsequent differences between the control and treated samples. Visually there is the suggestion of a rise in the treated samples, and using the non-parametric trend test for samples up to day 14 (as there is only one day 21 treated sample), this approaches significance ($z = 1.92$, $p < 0.06$), whereas for the control samples, there is no such trend ($z = 0.84$, $p > 0.4$).

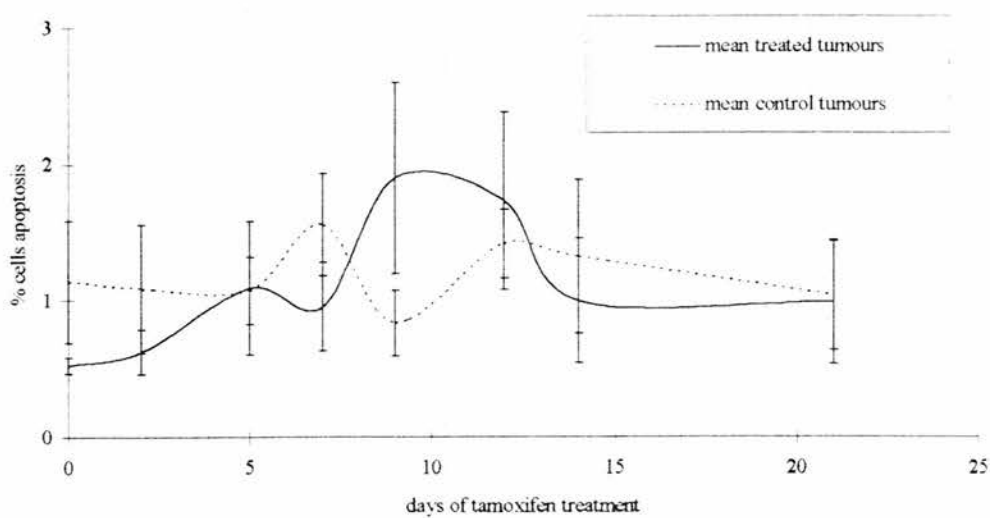


Figure 6.2.6 Apoptosis (using DNAase) in sequential FNAs from tamoxifen treated and control ZR-75-1 xenografts

Furthermore, as shown in table 6.2.1, there are excellent correlations between this method of assessing apoptosis and the original method which omits the DNAase step.

| all tumours | control tumours | treated tumours |
|--------------------------------|--------------------------|------------------------|
| $r_1 = 0.712$ ($p < 0.0001$) | 0.901 ($p < 0.0001$) | 0.498 ($p < 0.01$) |

Table 6.2.1 Correlations between the percentage of cells in apoptosis as measured on the FACScan, when analysed with and without pre-treatment with DNAase.

The Apoptotic:G₂/M ratio (calculated as the ratio between the proportion of cells in apoptosis and G₂/M) for tamoxifen treated and control ZR-75-1 xenografts is shown in figure 6.2.7. The values for the treated ZR-75-1 tumours are significantly higher than the control tumours on days 2 ,5 and 7($p < 0.05$), but not thereafter, when there are fewer data points.

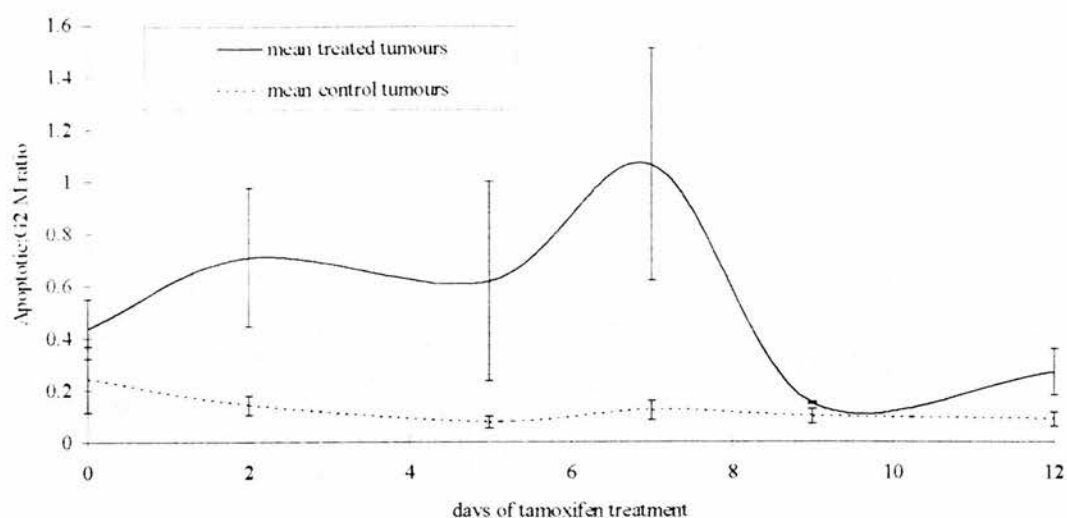


Figure 6.2.7 Apoptotic:G2/M ratio in sequential FNAs from tamoxifen treated and control ZR-75-1 xenografts

In contrast there appears to be a fall in the level of apoptosis (as detected by FACS-TUNEL) in the MDA-MB-231 tumours, irrespective of whether or not they had been treated with tamoxifen, as can be seen in figure 6.2.8. However this apparent trend is not significant, and indeed there are no significant differences between the treated and control tumours at any time point, confirming that tamoxifen does not have any effect upon the rate of apoptosis in MDA-MB-231 tumours.

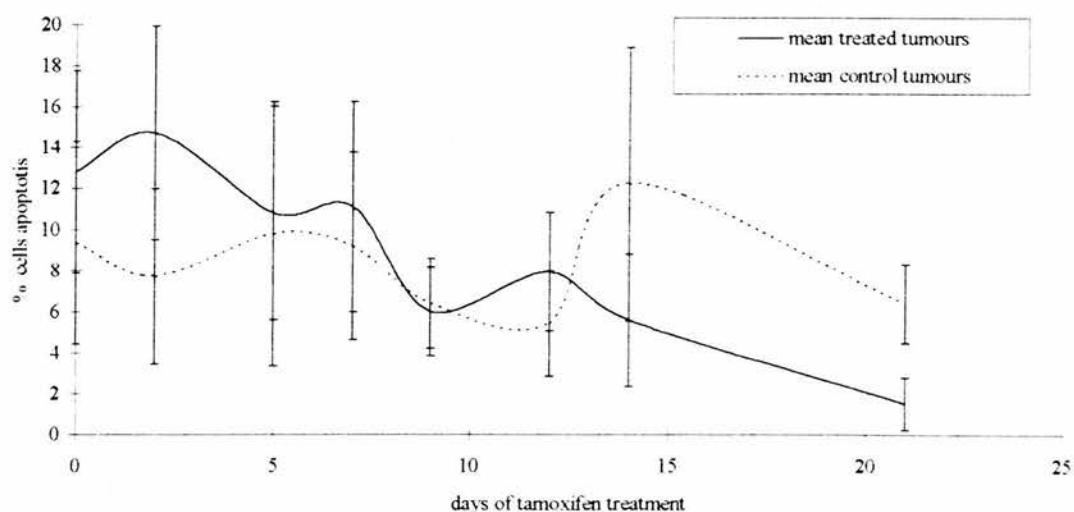


Figure 6.2.8 Apoptosis in sequential FNAs from tamoxifen treated and control MDA-MB-231 xenografts

Similarly, when considering the apoptotic:mitotic ratio in the treated and control MDA-MB-231 xenografts, figure 6.2.9 confirms that there are no differences between the values in the treated and control tumours. Consistent with the apparent fall in apoptosis in the treated tumours, a similar trend is seen for the Apoptotic:mitotic ratio, but it is not significant.

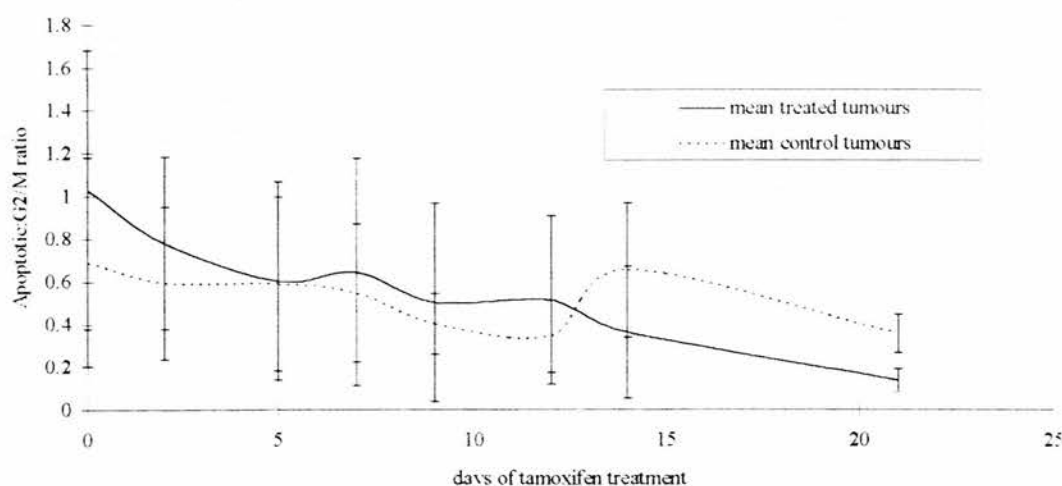


Figure 6.2.9 Apoptotic:G2/M ratio in sequential FNAs from tamoxifen treated and control MDA-MB-231 xenografts

The more important question is however whether the sequence of values for an individual tumour can identify it as a treated (and thus responding) or a control ZR-75-1 tumour. Clearly one approach would be to consider the regression lines of the $\log(\text{Apoptotic:G}_2/\text{M ratio})$: however for none of the treated tumours are these significantly different from horizontal, even if they do in most cases rise. In contrast the control tumours have horizontal or down-sloping lines, although again none are significantly different from the horizontal. Examples of the results for individual treated and control tumours are to be seen respectively in figures 6.2.10 and 6.2.11 (although note the different y-axis scales), where it can be seen that the variation in values for one tumour is too great to be able to distinguish treated from control tumours. Equivalent intra-tumour variation is seen in individual treated and untreated MDA-MB-231 tumours.

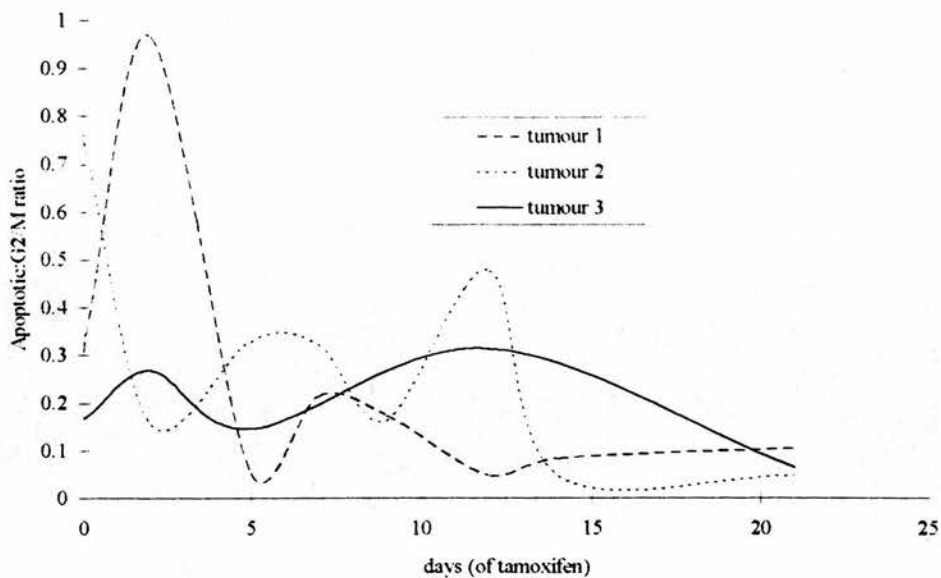


Figure 6.2.10 Apoptotic:G2/M ratio in individual tamoxifen treated ZR-75-1 xenografts subjected to sequential FNAs

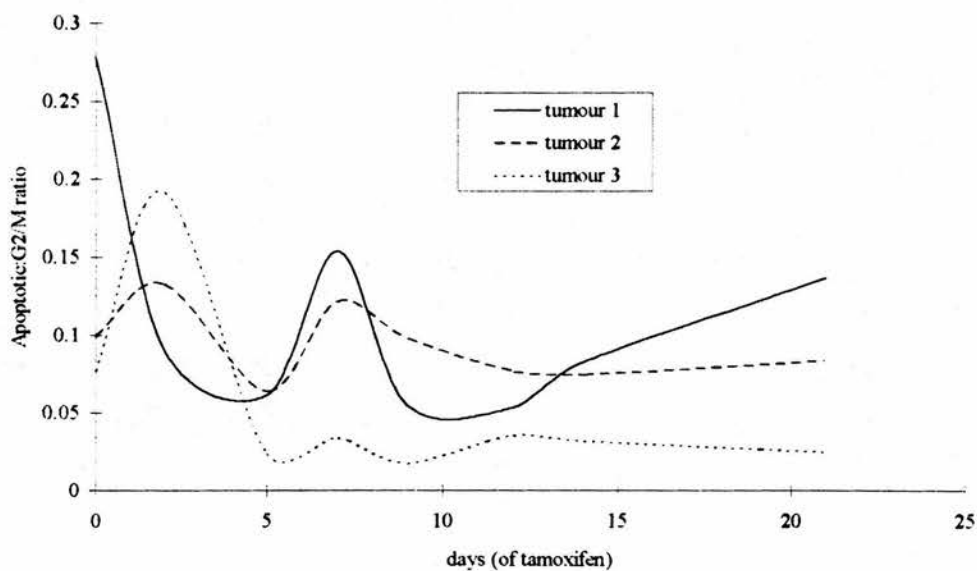


Figure 6.2.11 Apoptotic:G2/M ratio in individual control ZR-75-1 xenografts subjected to sequential FNAs

6.3 Discussion

The data obtained by subjecting individual tumours to sequential fine needle aspiration do not appear to clarify the timing of the previously noted changes in apoptosis and mitosis consequent upon tamoxifen treatment of the ZR-75-1 xenografts. It had been hoped that this experiment would indicate that either changes in proliferation, apoptosis or their ratio would serve as a marker sufficiently sensitive to be able to identify an individual tumour as responding to tamoxifen. Furthermore by taking samples at various time points it was anticipated that the group analysis would indicate more precisely the duration of the changes in apoptosis and mitosis. However the data remain entirely consistent with the results seen in sections 3 and 4 where data from separate tumours are pooled and compared.

Firstly figures 6.1.1 and 6.1.2, when taken into consideration with figures 3.1.1 and 4.1.1, strongly suggest that taking sequential FNAs in individual tumours does not profoundly affect their growth curves, nor the ability of tamoxifen to induce regression in the sensitive ZR-75-1 breast cancer cell line. Furthermore figures 6.2.1 and 6.2.2 confirm that tamoxifen has an anti-proliferative effect in these same tumours, and indeed when compared with figure 3.6.2, the difference between the treated and control tumours is more definite in this current experiment. Similarly for the changes in apoptosis, the treated tumours as a group do not appear to manifest a rise as compared to the initial day 0 value, but when comparing the values seen on days 2 - 7 with those in the control tumours, then there is a significant difference, which was not apparent in the prior experiment (see figure 3.6.1). Furthermore there are no differences between the treated and control MDA-MB-231 tumours, confirming the data in section 4 (see figures 4.6.1 and 4.6.2). Thus the changes induced by tamoxifen in the proportions of cells in the various stages of the cell cycle and/or apoptosis would appear to be better detected by the use of sequential FNAs than single FNAs in different tumours. However the pattern seen within one tumour shows too much variation to permit identification of the tumour as one that is responding to tamoxifen, as figures 6.2.10 & 6.2.11 demonstrate.

Consideration of figure 6.2.5 suggests that the rise in apoptosis induced by tamoxifen is not sustained beyond day 7, as was also seen in figure 3.2.1, whereas the anti-proliferative effect appears to be prolonged.

7. Analysis of individual ZR-75-1 and MDA-MB-231 Xenografts

The data presented from the xenografts in sections 3 - 6 have concentrated on the results of analysing the tumours in groups, usually on the basis of the time that the tumour was excised (or subjected to an FNA), and whether or not it had been treated with tamoxifen. However, as has already been noted, not all tumours of the same cell line behaved in an identical manner. For example, 4/10 of the ZR-75-1 xenografts that were untreated and excised on day 28 actually regressed between days 21 & 28, in contrast to their prior growth and the behaviour of the other 6. Equally the MDA-MB-231 tumours from passage 2 that had been left untreated and excised on day 28, had smaller volumes, and statistically different mitotic indices and apoptotic:mitotic ratios as compared with their untreated counterparts removed on day 21 at the end of passage 7. Further evidence for considerable intra-tumour variation in proliferation and apoptosis comes is seen in figures 6.2.10 and 6.2.11, where the changes in the apoptotic:mitotic ratio within a single xenograft are such that the trend over time cannot be used to distinguish clearly a tamoxifen treated from an untreated tumour. Hence it is appropriate to consider the individual behaviour of all these xenografts, both in terms of their growth and its relation to measurements made such as the apoptotic and mitotic indices, necrosis and flow cytometric data.

This section will therefore examine individual tumour growth; how it can be modelled and/or predicted by the Gompertz function, and the conclusions that can be drawn from analysing data pertaining to individual tumours.

7.1 Growth of ZR-75-1 xenografts

Although tamoxifen treated tumours as a group clearly regressed from day 14 onwards (see figure 3.1.1), individual tumours regressed at varying time points, as illustrated in table 7.1.1 where it can be seen that even after 28 days' tamoxifen, 2 treated tumours had not regressed. In particular, on day 14 when the volumes as a group were significantly less than for the untreated tumours, more than half the excised tumours (and upon which the histological and flow cytometric data were recorded) were still larger than they had been at the time tamoxifen therapy was commenced. The

equivalent data for the day 28 control tumours is shown in table 7.1.2, which confirms that a proportion of the day 28 tumours had started to regress before the end of the experiment.

| day | 2 | 7 | 14 | 21 | 28 |
|-------------|-------|-------|-------|------|------|
| all treated | 64/64 | 39/62 | 16/50 | 5/39 | 2/28 |
| excised | 12/12 | 6/12 | 5/11 | 2/11 | 2/28 |

Table 7.1.1 Numbers of tamoxifen treated ZR-75-1 xenografts that were still larger than they had been on day 0.

| day | 2 | 7 | 14 | 21 | 28 |
|-------------------------|----|----|----|----|----|
| day 28 control (n = 10) | 10 | 10 | 10 | 9 | 6 |

Table 7.1.2 Numbers of untreated ZR-75-1 xenografts that were still larger than they had been at the previous time point.

This point is further illustrated by figure 7.1.1, where it can be seen that the volumes of the tumours actually excised at each time point are not necessarily representative of those of the tumours that remain on treatment ; the volumes of the excised tumours are significantly larger than the remaining treated tumours on day 2 ($p < 0.02$), with trends on days 0 ($p = 0.1$) and day 7 ($p = 0.08$).

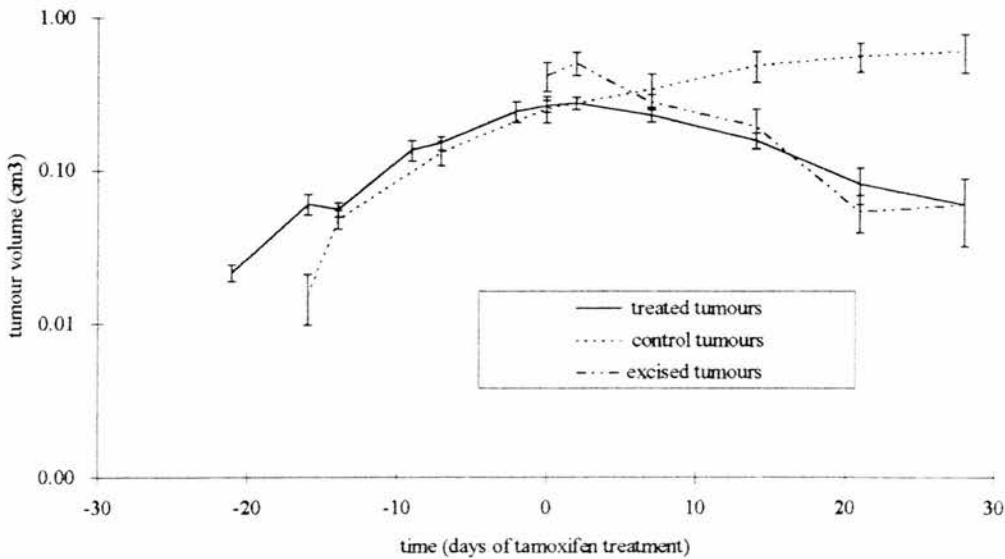


Figure 7.1.1. Growth of control and tamoxifen treated ZR-75-1 xenografts

With these differences in growth in mind, tumour volumes from individual xenografts were fitted to a Gompertz function. Assessment of goodness-of-fit can be done in different ways, since there are inevitable errors in the tumour measurements from which the volumes are calculated. Residuals (the terminology is taken from linear regression analysis) refer to the difference between the actual measured volume, and that predicted by the model function. Thus one method of assessing the goodness-of-fit of Gompertzian growth is to use the residuals to compare actual with predicted growth, but this can only be done for the untreated tumours. Table 7.1.3 gives the average residual for the day 28 control tumours, where it can be seen that the data suggest that the prediction up to day 14 is good, but thereafter becomes less accurate. Figures 7.1.2 and 7.1.3 respectively illustrate a tumour with excellent and poor prediction of growth, but it needs to be recalled that 4/10 of these control tumours regressed between days 21 & 28, and one indeed started to regress between day 14 & 21. Furthermore, tumour DAC051 had no growth between day 21 & 28, so it is perhaps less surprising that of the non-regressing controls, the growth curve based on the volumes up to day 0 appears to fit least well for this tumour. The cause of the regression seen in some of these control tumours is not apparent, but it is therefore no great surprise that the Gompertz function becomes a less accurate predictor of growth beyond day 14, suggesting that there was a fundamental change in the control tumour growth towards the end of the experiment.

| volumes used | Average residual for predicted volumes | | | | |
|---------------------------|--|--|--------------|--------------|-------------|
| | up to day 0 (predicting up to day 28) | up to day 0 (predicting only to day 14) | up to day 14 | up to day 21 | all volumes |
| DAC050 | 0.24 | 0.11 | 0.016 | 0.041 | 0.0056 |
| DAC051 | 2.63 | 0.74 | 0.013 | 0.023 | 0.0093 |
| DAC053 | 0.042 | 0.0090 | 0.046 | 0.049 | 0.0055 |
| DAC056 | inadequate data | | 0.15 | 0.00042 | 0.013 |
| DAC058 | inadequate data | | | | |
| DAC059 | 0.32 | 0.42 | 0.31 | 0.14 | 0.030 |
| Regressing tumours | | | | | |
| DAC052 | 2.35 | 0.46 | 0.76 | 1.14 | 0.11 |
| DAC087 | 1.9 | 2.2 | 2.2 | 1.2 | 0.14 |
| DAC082 | 0.40 | 0.26 | 0.44 | 0.42 | 0.036 |
| DAC096 | 0.36 | 0.14 | 0.57 | 0.42 | 0.066 |

Table 7.1.3. Average residuals for day 28 control ZR-75-1 xenograft tumours, depending on the volumes to which the Gompertz function was fitted.

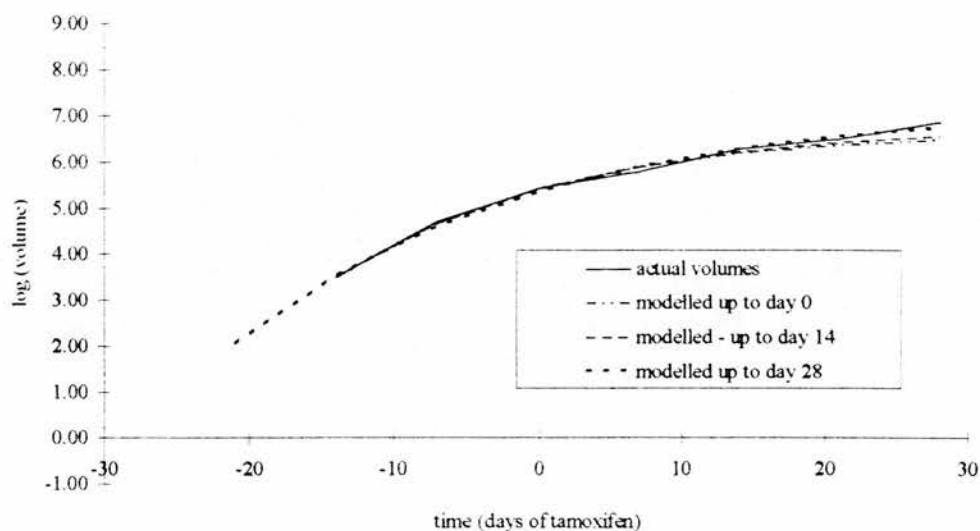


Figure 7.1.2 DAC053 - a control ZR-75-1 tumour with well predicted growth curves

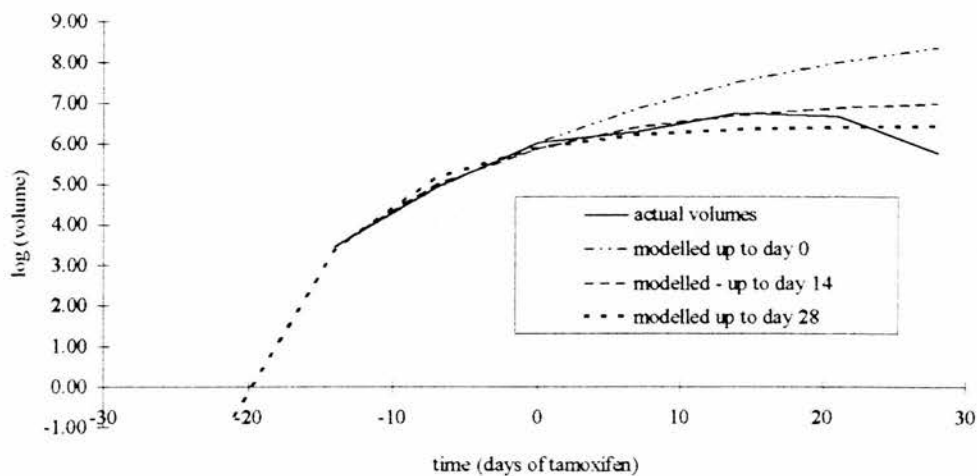


Figure 7.1.3 DAC052 - a control ZR-75-1 tumour with poorly predicted growth curves

There is another way to assess the ability of the Gompertz growth function to model subsequent tumour growth, again comparing actual and predicted growth. Since the tumour volumes are all log-normally distributed, one can use the student t-test to consider the null hypothesis that there are no differences between the actual and

predicted volumes of control tumours. Table 7.1.4 shows the results of these tests, and with the significance level set at $p < 0.01$ (because of multiple comparisons) there are no statistically significant differences.

| Gompertz curve fitted using volumes up to | actual vs predicted on | | | | |
|---|------------------------|-------|--------|--------|--------|
| | day 0 | day 7 | day 14 | day 21 | day 28 |
| day 0 | n.s. | n.s. | n.s. | n.s. | n.s. |
| day 7 | | n.s. | n.s. | n.s. | n.s. |
| day 14 | | | n.s. | n.s. | n.s. |
| day 21 | | | | n.s. | n.s. |
| day 28 | | | | | n.s. |

Table 7.1.4. Student t-test comparisons between actual and predicted growth curves for untreated ZR-75-1 xenografts.

The same methodology can be used to compare predicted and actual tumour volumes in the tamoxifen treated tumours ; and as it is shown in table 7.1.5, significant differences are detected at day 7 and beyond. It should be recalled from section 3.1 that comparing the treated tumour volumes with the control volumes, no significant differences were detected until day 14. Thus table 7.1.5 suggests that the use of a predicted growth curve for an individual tumour might be a more sensitive way to detect alterations in tumour growth than a comparison with untreated growth in a different tumour, in which there might be other, unspecified, differences in growth-regulating factors.

| actual vs predicted volumes on | treated tumours excised on day | | | | | |
|--------------------------------|--------------------------------|------|-------------|-------------|-------------|-------------|
| | 0 | 2 | 7 | 14 | 21 | 28 |
| day 0 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| day 2 | | n.s. | n.s. | n.s. | n.s. | n.s. |
| day 7 | | | $p < 0.002$ | $p < 0.01$ | n.s. | $p < 0.001$ |
| day 14 | | | | $p < 0.002$ | $p < 0.01$ | $p < 0.001$ |
| day 21 | | | | | $p < 0.002$ | $p < 0.001$ |
| day 28 | | | | | | $p < 0.001$ |

Table 7.1.5. Student t-test comparisons between actual and predicted growth curves for tamoxifen treated ZR-75-1 xenografts, fitting a Gompertz curve to the pre-treatment volumes.

Implicit in all the analysis of growth and regression is the assumption that there are no differences in tumour behaviour dependent upon the particular flank of the animal into

which the initial fragment was inserted. Previous reports suggest that for Gompertzian growth the maximum tumour volume ($e^{a/b}$) is animal (species) specific (Brunton et al(1978)). One would expect therefore that the parameters for the tumours would be independent of the side of the animal into which they were implanted ; this is in fact the case for a, b, their ratio a/b and the "time from" parameter. Indeed only 10/37 pairs of tumours had any significant difference between the a/b ratios of the two tumours.

7.2 Growth of MDA-MB-231 xenografts

The first analysis of individual tumour growth in the MDA-MB-231 xenografts is to compare for each time point the volumes of the excised tumours with the remaining treated tumours. Figure 7.2.1 illustrates, that in contrast to the ZR-75-1 xenografts, there are no significant differences for the MDA-MB-231 tumours. Similarly individual growth comparisons between time points can be made ; there was 1 tamoxifen treated tumour that was smaller when excised (on day 14) than it had been on day 0 (having regressed from day 2 onward), and 1 further treated tumour that regressed between days 14 & 21. There was also one untreated tumour that regressed between days 14 & 21, and another that was smaller when excised on day 28 than it had been on day 0 ! Hence there was variation in individual tumour growth for the MDA-MB-231 xenografts.

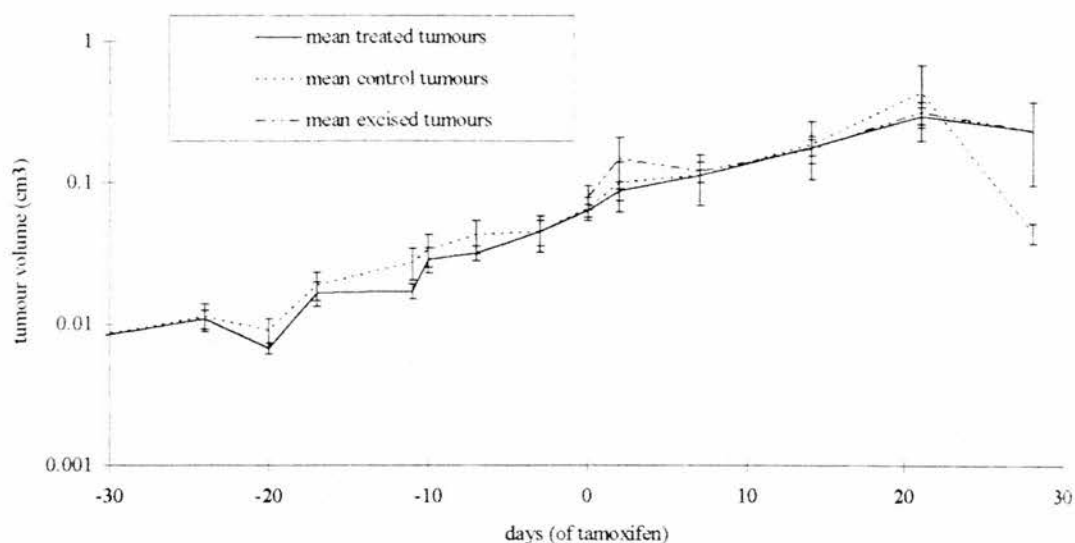


Figure 7.2.1 Growth data for tamoxifen treated and control MDA-MB-231 xenografts

As for the ZR-75-1 xenografts, individual tumour growth was fitted to a Gompertz function. Comparison of actual and predicted growth was assessed using the same two methods as for the ZR-75-1 xenografts. Table 7.2.1 gives the average residual for the control tumours, using the volumes only up to day 0. It can clearly be seen that in general the Gompertz curve provides a much better fit over the duration of growth for the control tumours than was seen in the ZR-75-1 tumours, particularly in the day 28 control tumours from passage 2.

| volumes used | Average residual for predicted volumes | | |
|-----------------|--|--------------|-------------|
| | up to day 0 | beyond day 0 | all volumes |
| Day 21 controls | | | |
| DAC243 | 0.0021 | 0.293 | 0.207 |
| DAC247 | 0.005 | 0.061 | 0.043 |
| DAC252 | 0.0034 | 0.279 | 0.211 |
| DAC256 | 0.0077 | 0.651 | 0.460 |
| DAC260 | inadequate data | | |
| Day 28 controls | | | |
| DAC201 | 0.00001 | 0.00012 | 0.00007 |
| DAC205 | < 0.00001 | 0.00002 | 0.00001 |
| DAC222 | < 0.00001 | 0.00046 | 0.00031 |
| DAC223 | < 0.00001 | 0.00147 | 0.00084 |

Table 7.2.1. Average residuals for day 21 & day 28 control MDA-MB-231 xenograft tumours.

As with the ZR-75-1 tumours, one can use the student-t test to consider the null hypothesis that there are no statistically significant differences between the (logarithms of) the actual and Gompertz-predicted tumour volumes, as seen for the control tumours, in table 7.2.2, and the tamoxifen treated xenografts in table 7.2.3. In both cases there are no overall significant differences, showing that not only does the Gompertz function predict subsequent growth well, but confirming that tamoxifen treatment does not alter the fundamental growth of the MDA-MB-231 tumours. The single significant exception, the day 28 tumours on day 14, is probably a chance event as the same trend is not seen in other tumours on day 14, nor in the same tumours at other time points.

| actual vs predicted using volumes up to : | control tumour volumes on | | | | |
|--|---------------------------|-------|--------|--------|--------|
| | day 0 | day 7 | day 14 | day 21 | day 28 |
| day 0 | n.s. | n.s. | n.s. | n.s. | n.s. |
| day 7 | | n.s. | n.s. | n.s. | n.s. |
| day 14 | | | n.s. | n.s. | n.s. |
| day 21 | | | | n.s. | n.s. |
| day 28 | | | | | n.s. |

Table 7.2.3. Student t-test comparisons between actual and predicted growth curves for untreated MDA-MB-231 xenografts.

| actual vs predicted volumes on | treated tumours excised on day | | | | | |
|--------------------------------|--------------------------------|------|------|------|------|----------|
| | 0 | 2 | 7 | 14 | 21 | 28 |
| day 0 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| day 2 | | n.s. | n.s. | n.s. | n.s. | n.s. |
| day 7 | | | n.s. | n.s. | n.s. | n.s. |
| day 14 | | | | n.s. | n.s. | p < 0.01 |
| day 21 | | | | | n.s. | n.s. |
| day 28 | | | | | | n.s. |

Table 7.2.4. Student t-test comparisons between actual and predicted growth curves for tamoxifen treated MDA-MB-231 xenografts.

7.3. Correlations between individual tumour growth and biology

The important associations in this study are between tumour growth or regression, and the changes in the tumour biology. Detailed relationships between the biological parameters for groups of tumours have been reviewed in section 3.7, and thus this section will concentrate on the relationships between the biology and relative tumour size. Given that the majority of the ZR-75-1 tumours regressed when treated with tamoxifen, one needs to consider different measures of this regression. The three that will be considered are the actual tumour volume, the relative volume (as compared with the volume on day 0), and the response, defined as in the introduction (see page 40), namely the proportional reduction from the predicted volume at the same time-point, using a Gompertz growth curve fitted to the pre-treatment volumes for that individual tumour. In order to reduce the possibility of reporting false positive correlations, those significant at the 5% level are excluded unless there is another significant result for a similar variable. Table 7.3.1 reports the correlations between proliferative markers and tumour volume/response, and it can be seen in general that although there are no significant relationships for the untreated tumours, there is a significant association in the treated tumours such that higher proliferative rates are seen in the larger tumours (as evidence by higher actual or relative volumes), whereas negative correlations are seen with response. MIB-1 expression and S-phase fraction give consistent significant correlations - the mitotic index does not correlate with the relative volume and no significant relationships are seen with the proportion of cells in G2/M.

| | tumours | Cell growth | | | |
|-----------------|---------|-------------------|-------------------|------------------|--------------|
| | | MIB-1 | MI | S-phase | G2/M |
| response | treated | -0.463 < 0.001 | -0.477 < 0.001 | -0.488 < 0.01 | -0.304 ns |
| | control | ns | ns | ns | ns |
| relative volume | treated | 0.65 < 0.0001 | ns | 0.402 < 0.01 | ns |
| | control | ns | ns | ns | ns |
| actual volume | treated | 0.565 < 0.0001 | 0.505 < 0.0001 | 0.408 < 0.01 | ns |
| | control | ns | ns | ns | ns |

Table 7.3.1 Correlations between proliferation and tumour growth/regression for ZR-75-1 xenografts.

| Cell Death | tumours | Cell death | | | |
|-----------------|---------|------------|------------------|--------------------|------------------|
| | | log(A/M) | AI | necrosis | FACS TUNEL |
| response | treated | ns | -0.294 < 0.02 | -0.608 < 0.0001 | ns |
| | control | ns | ns | ns | ns |
| relative volume | treated | ns | 0.381 < 0.01 | 0.596 < 0.001 | 0.388 < 0.01 |
| | control | ns | -0.727 < 0.05 | ns | -0.686 < 0.05 |
| actual volume | treated | ns | 0.554 < 0.001 | 0.628 < 0.001 | ns |
| | control | ns | ns | ns | ns |

Table 7.3.2 Correlations between cell death and tumour growth/regression for ZR-75-1 xenografts.

In contrast table 7.3.2 reveals that there are fewer significant relationships between tumour volume/response and indicators of cell death. The apoptotic index correlates with all three measures of tumour growth in treated tumours, as does the necrotic proportion of the tumour. Again positive correlations are seen with volume and relative volume, whereas negative ones are seen with response. However given the previously noted positive associations between proliferation and both necrosis and apoptosis (see section 3.7), these observations are no more than consistent with table 7.3.1. The flow cytometric measure of apoptosis only correlates with the relative volume, and the Apoptotic:Mitotic ratio correlates with none of the three. For the untreated tumours, there are significant negative correlations between the relative volume and apoptosis as

measured by histology and flow cytometry. Figure 7.3.1 illustrates the data for the apoptotic index, and highlights the regressing tumours. (It should be noted that the relative volume is always 1 for the day 0 control tumours and therefore they are excluded from any analysis of a relationship between the relative volume and other data.) The data suggest that higher levels of apoptosis are associated with a smaller relative increase in volume, as would be anticipated as apoptosis is one of the mechanisms for cell loss during tumour growth. It can be seen that the explanation for this negative trend is not just that four of the tumours had started to regress, and it should be recalled that the Gompertz growth curves suggest that in general this group of control tumours had undergone a change in overall growth behaviour before the end of the experiment. Given that the oestrogen pellets, essential for the growth of these xenografts, were nearing the end of their 60-day life, it is possible that the explanation for the regression and the negative correlation between apoptosis and relative volume was due to effective oestrogen withdrawal. The absence of a significant relationship between the apoptotic index and the response could be due to the previously demonstrated divergent trend between Gompertz-predicted and actual tumour volumes beyond day 14 ; recalling that the response is defined using the Gompertz curve fitted up to day 0 only.

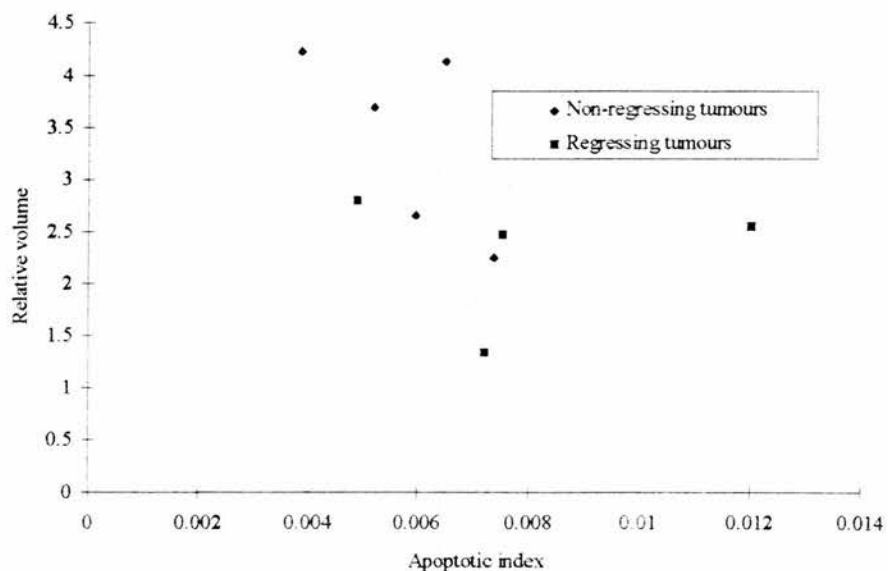


Figure 7.3.1 Apoptotic index and relative volume in day 28 control ZR-75-1 xenografts

However, the aim of this study was primarily to detect changes that are associated with, and might therefore be predictive of, tamoxifen sensitivity. By volume comparisons, tamoxifen only had significant growth effects by day 14, although Gompertz growth curve deviation was significant for most tumours by day 7 (see section 7.1). Therefore table 7.3.3 lists the significant relationships at these early time points. It can be seen on day 7, when the group analysis demonstrated that apoptosis had risen and proliferation fallen in the treated tumours, there are significant relationships such that the apoptotic:mitotic ratio, the apoptotic index and MIB-1 expression all correlated with the response. It should be recalled that day 7 is also the first day at which Gompertz growth curves could detect a significant change in growth in these same tumours. Relative volume, a cruder indicator of the change in volume with treatment, also correlated with the apoptotic:mitotic ratio and the proportion of cells expressing MIB-1. On day 14, the relationships become weaker for response, although there are significant correlations with the mitotic index. In the control tumours there are no relationships with MIB-1 expression, but there are with apoptotic index (which has already been previously commented upon for relative volume).

| | | MIB-1 | M.I. | A.I. | log(A/M) |
|--------------------------------|------------------------|-------------------|------------------|------------------|-------------------|
| day 0 (n=13) | all measures | ns | ns | ns | ns |
| day 2 (n=12) | all measures | ns | ns | ns | ns |
| day 7 (n=12) | volume | ns | ns | ns | ns |
| | response | -0.886 < 0.002 | ns | 0.72 < 0.02 | 0.944 < 0.001 |
| | relative volume | 0.671 < 0.05 | ns | ns | 0.799 < 0.01 |
| day 14 (n=11) | volume | ns | ns | ns | ns |
| | response | ns | -0.656 < 0.05 | ns | 0.569 < 0.1 |
| | relative volume | 0.845 < 0.002 | 0.791 < 0.01 | ns | 0.632 p < 0.05 |
| day 28 control (n=9) | volume | ns | ns | -0.633 < 0.1 | ns |
| | response | ns | ns | 0.786 < 0.02 | ns |
| | relative volume | ns | ns | -0.767 < 0.02 | -0.667 < 0.05 |

Table 7.3.3 Correlations between markers of proliferation and apoptosis in individual ZR-75-1 xenografts removed at different time points.

Figure 7.3.2 illustrates the correlation on day 7 between the apoptotic:mitotic ratio and response, and that for MIB-1 expression is shown in figure 7.3.3. It should be noted that a negative value for response indicates that the tumour volume on day 7 is larger than that predicted for by the Gompertz growth curve, and correspondingly values of response that are greater than zero reflect increasing degrees of tumour regression. Hence we see that the more a tumour has fallen away from its predicted Gompertz growth curve, the lower its proliferation (as expressed by MIB-1) and the higher the apoptotic:mitotic ratio.

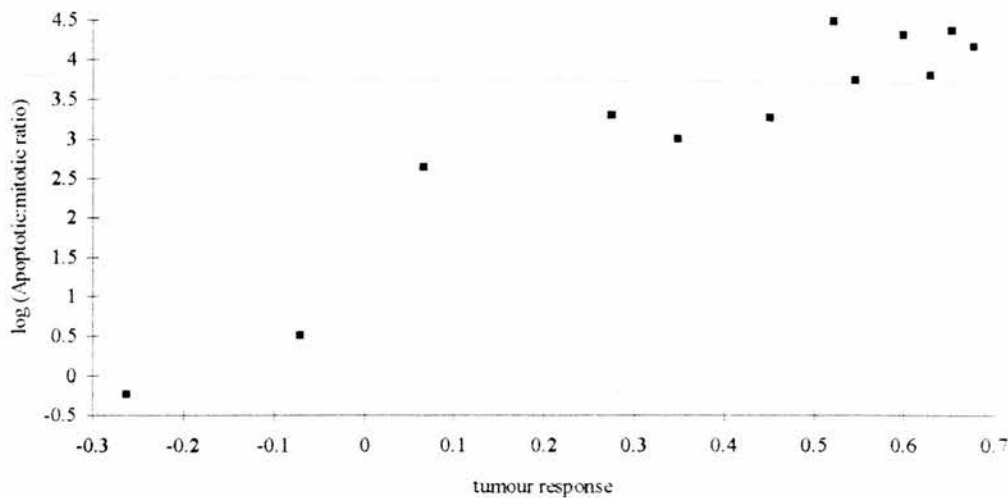


Figure 7.3.2 Apoptotic : Mitotic ratio and tumour response in ZR-75-1 xenografts after 7 days' tamoxifen treatment

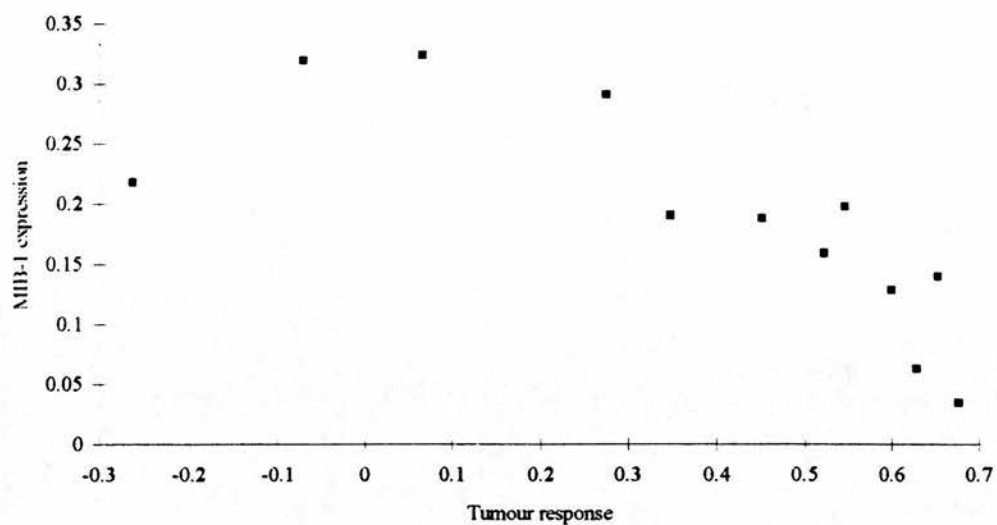


Figure 7.3.3 MIB-1 expression and tumour response in ZR-75-1 xenografts after 7 days' tamoxifen

In contrast it should be noted that for the treated MDA-MB-231 xenografts there are no significant relationships between on the one hand volume, response and relative volume and on the other apoptosis, mitosis or their ratio.

7.4 Synchronously borne ZR-75-1 and MDA-MB-231 tumours

Insufficient tumour measurements had been taken prior to the insertion of tamoxifen pellets in this experiment, so that not all tumours had sufficient volume data to which a Gompertz growth curve could be fitted. Table 7.4.1 reports the available data, and it can be seen that on average only about half the tumours could be fitted with an individualised growth curve.

| | control tumours | | treated tumours | |
|-------|-----------------|---------|-----------------|---------|
| | MDA-MB-231 | ZR-75-1 | MDA-MB-231 | ZR-75-1 |
| day 0 | 2/4 | 2/4 | | |
| day 2 | 4/5 | 2/5 | 4/5 | 5/5 |
| day 7 | 2/5 | 3/5 | 3/5 | 1/5 |

Table 7.4.1 Tumours with sufficient pre-treatment volumes for Gompertz growth curve fitting.

The paucity of data makes it impossible to use the difference between actual and predicted growth to determine whether, as was seen in sections 7.1 & 7.2, Gompertz curve analysis could detect tamoxifen-induced changes in the growth of the treated ZR-75-1 but not MDA-MB-231 xenografts. However the average residuals can still be determined, and they are shown in table 7.4.2. They suggest that only the treated ZR-75-1 and untreated MDA-MB-231 tumours deviate substantially from the predicted growth curves. However the latter comment is partly due to a single regressing control MDA-MB-231 tumour, which interestingly was borne by the same animal as the only regressing untreated ZR-75-1 tumour - and this may well therefore reflect a problem with that particular mouse rather than the MDA-MB-231 tumours as a whole.

| | control tumours | | treated tumours | |
|-------|--|---------|-----------------|----------------------|
| | MDA-MB-231 | ZR-75-1 | MDA-MB-231 | ZR-75-1 |
| day 2 | 0.011 | 0.0066 | 0.0013 | 0.0062 |
| day 7 | 0.41 (0.002 without 1 regressing tumour) | 0.021 | 0.0098 | 0.12 (1 tumour only) |

Table 7.4.2 Average residuals for MDA-MB-231 and ZR-75-1 xenografts.

One way around the lack of individual tumour volume data is to use the average volumes of the various groups of tumours before day 0, and derive a Gompertz growth

curve from them. Figures 7.4.1 - 7.4.4 illustrate that this can be done for the treated and untreated tumours as a whole, for both ZR-75-1 and MDA-MB-231 xenografts, with no discernible effect on growth being evident as a consequence of tamoxifen therapy. Furthermore there are no statistically significant differences between the actual and predicted volumes for any group of tumours.

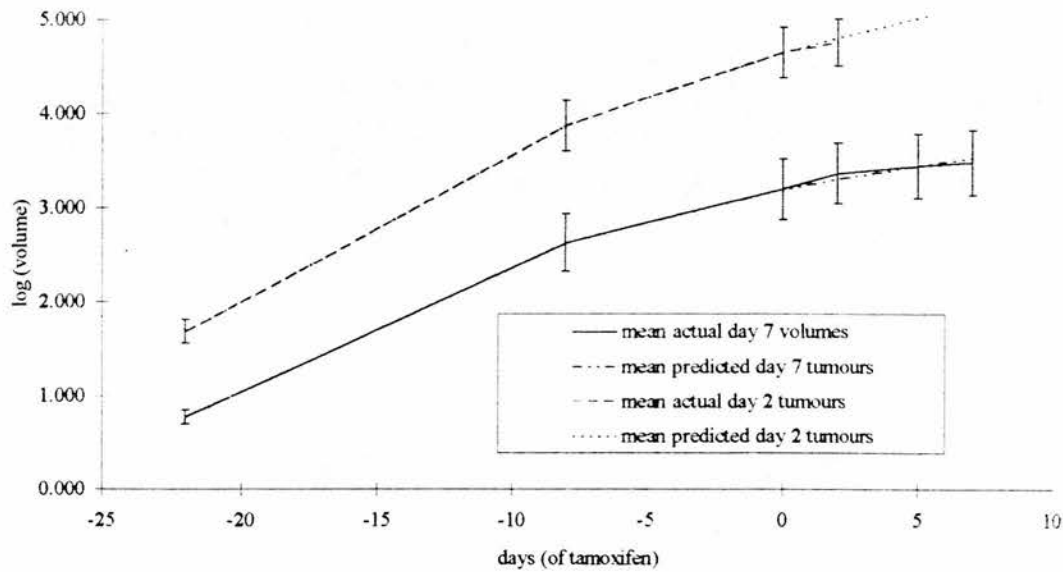


Figure 7.4.1 Predicted and actual growth curves for treated ZR-75-1 xenografts

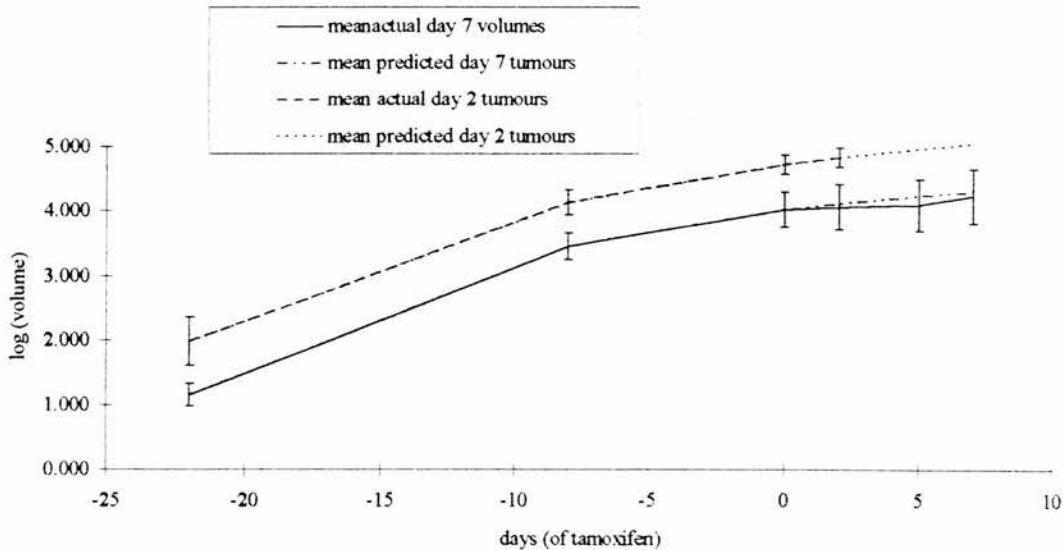


Figure 7.4.2 Predicted and actual growth curves for treated MDA-MB-231 xenografts

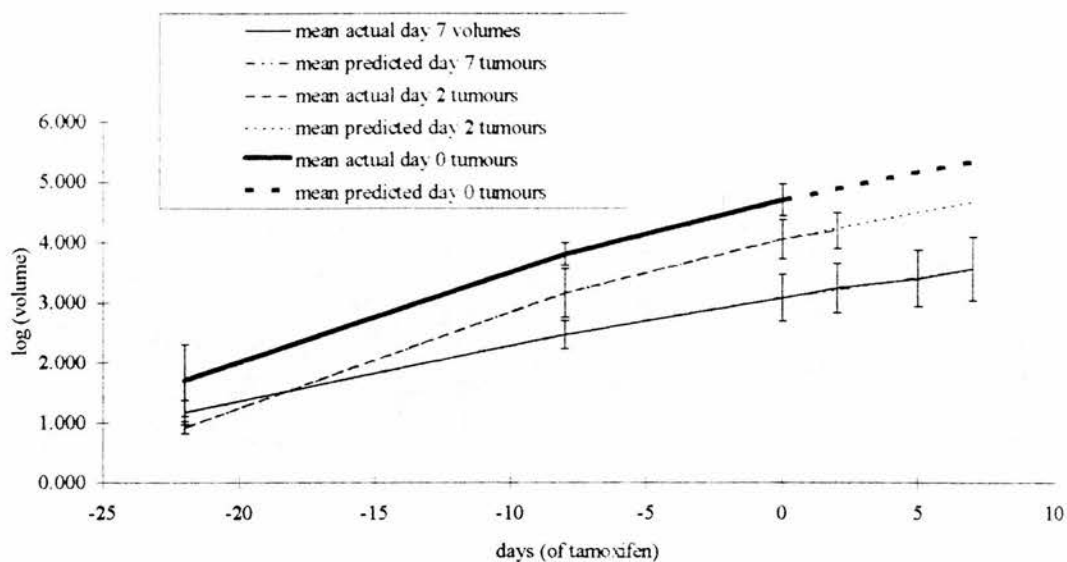


Figure 7.4.3 Predicted and actual growth curves for control ZR-75-1 xenografts

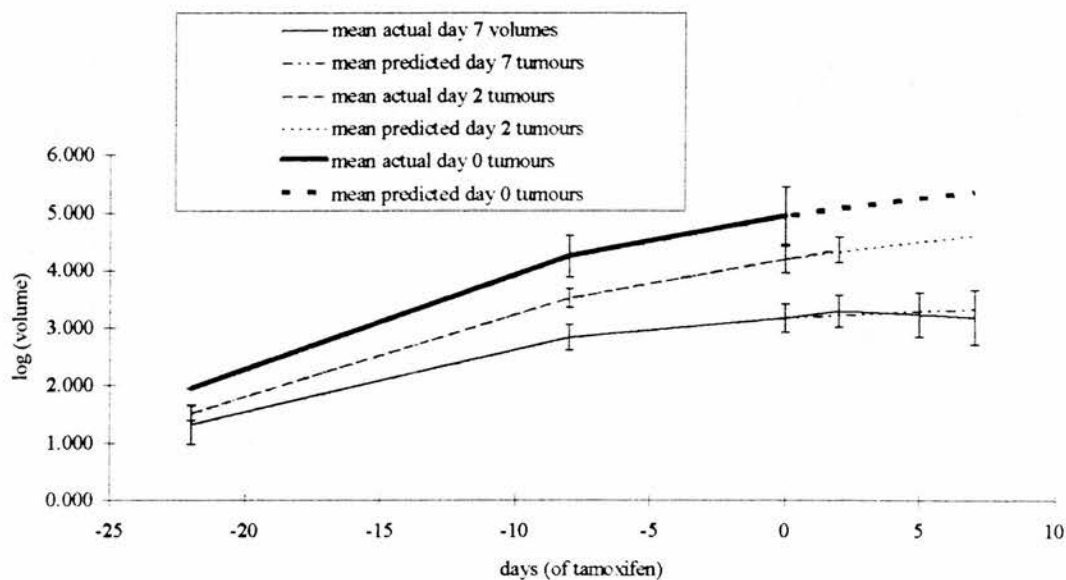


Figure 7.4.4 Predicted and actual growth curves for control MDA-MB-231 xenografts

These "average" Gompertz growth curves can be looked at in slightly more detail by considering their growth parameters. Table 7.4.3 demonstrates that although the two types of tumours appear to have begun growing at more or less identical times (as evidenced by the similarity of their "time from" parameters), consistent with their identical implantation dates, they do not follow identical growth curves, with the MDA-MB-231 tumours having smaller maximum volumes. With a only small number of tumours in this experiment it is not possible to say whether or not this represents fundamental differences in the growth determinants of these tumours.

| | ZR-75-1 tumours | | | MDA-MB-231 tumours | | |
|-------------|-----------------|---------|--------|--------------------|---------|--------|
| | treated | control | day 0 | treated | control | day 0 |
| V_{max} | 0.218 | 0.872 | 0.871 | 0.176 | 0.0881 | 0.289 |
| alpha | 0.2559 | 0.1786 | 0.3043 | 0.3536 | 0.2740 | 0.4882 |
| beta | 0.0475 | 0.0264 | 0.0450 | 0.0684 | 0.0612 | 0.0851 |
| "time from" | 4.45 | 5.31 | 3.33 | 4.29 | 5.2 | 1.3 |

Table 7.4.3 Growth parameters for Gompertz growth curves fitted to average volumes for MDA-MB-231 and ZR-75-1 xenografts.

It has been previously noted that for the MDA tumours there were no correlations between the apoptotic and mitotic indices and actual or predicted tumour volumes, and similarly there was no relationship between the apoptotic:mitotic ratio and tumour volumes. In contrast, relationships were found for the treated ZR-75-1 tumours (see section 7.3), with the apoptotic:mitotic ratio being correlated with the response on days 7 & 14.

In this experiment there were inadequate data to fit individual growth curves. Therefore a similar relationship between response or relative volume and the apoptotic:mitotic index in individual tumours cannot be determined. However one could use "average" Gompertzian growth curves, fitted to the average of all ZR-75-1 tumour volumes in this experiment, normalised to the day 0 volume of the individual tumours. In this case, as figure 7.4.5 shows, the 4 tamoxifen-treated ZR-75-1 tumours excised on day 7 closely fit the regression line derived for the equivalent tumours in the previous experiment (as described in section 3.7). Although visually these four tumours appear to demonstrate a similar relationship between response and their apoptotic:mitotic ratio, this does not approach statistical significance.

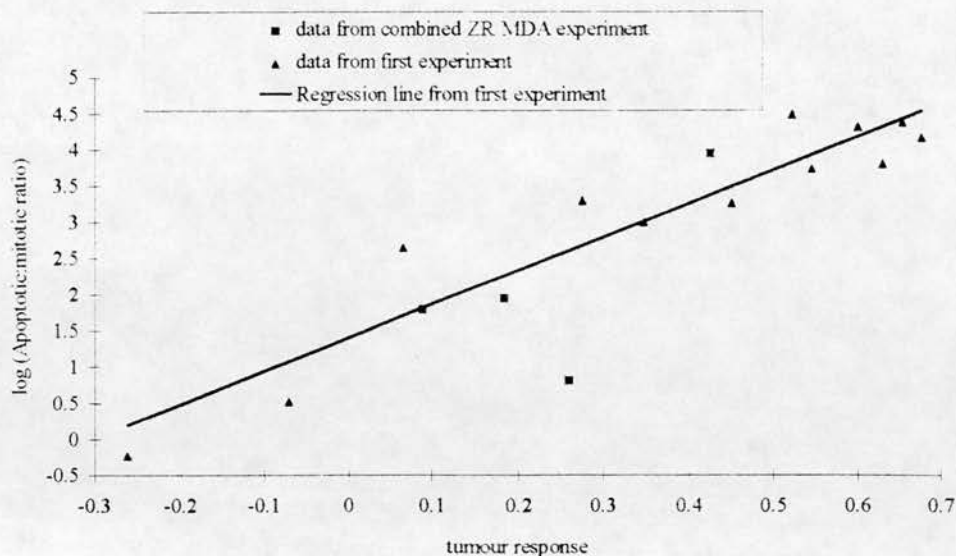


Figure 7.4.5 Apoptotic : Mitotic ratio and tumour response in all ZR-75-1 xenografts after 7 days' tamoxifen treatment

7.5 ZR-75-1 and MDA-MB-231 tumours subjected to sequential FNAs

For the tumours subjected to sequential FNAs, there were again insufficient data points to undertake individual Gompertz growth curve fitting. Therefore the average volumes for the four groups of tumours were used, and the Gompertz growth curve parameters are shown in table 7.5.1. This confirms that the ZR-75-1 and MDA-MB-231 tumours do grow differently, with as in the previous experiment, larger maximum volumes being seen for the ZR-75-1 xenografts. It should be recalled that the tumours in both this experiment and that immediately before were all established at the same passage. An understandable similarity, particularly for the MDA-MB-231 tumours, then becomes apparent between the figures in tables 7.5.1 and 7.4.3.

| | ZR-75-1 | | MDA-MB-231 | |
|-------------|---------|---------|------------|---------|
| | treated | control | treated | control |
| V_{max} | 2.252 | 3.971 | 0.105 | 0.105 |
| alpha | 0.1967 | 0.1572 | 0.4823 | 0.4921 |
| beta | 0.02548 | 0.01897 | 0.1036 | 0.1057 |
| "time from" | 5.23 | 7.11 | 3.60 | 2.31 |

Table 7.5.1 Growth parameters for Gompertz growth curves fitted to average volumes for MDA-MB-231 and ZR-75-1 xenografts subjected to sequential FNAs.

These average predicted growth curves are demonstrated in figures 7.5.1 and 7.5.2, where it can be seen that the only group of tumours to significantly deviate from the predicted growth curve are the ZR-75-1 treated tumours, and that the predicted curves are very similar for the treated and untreated groups of tumours of each type.

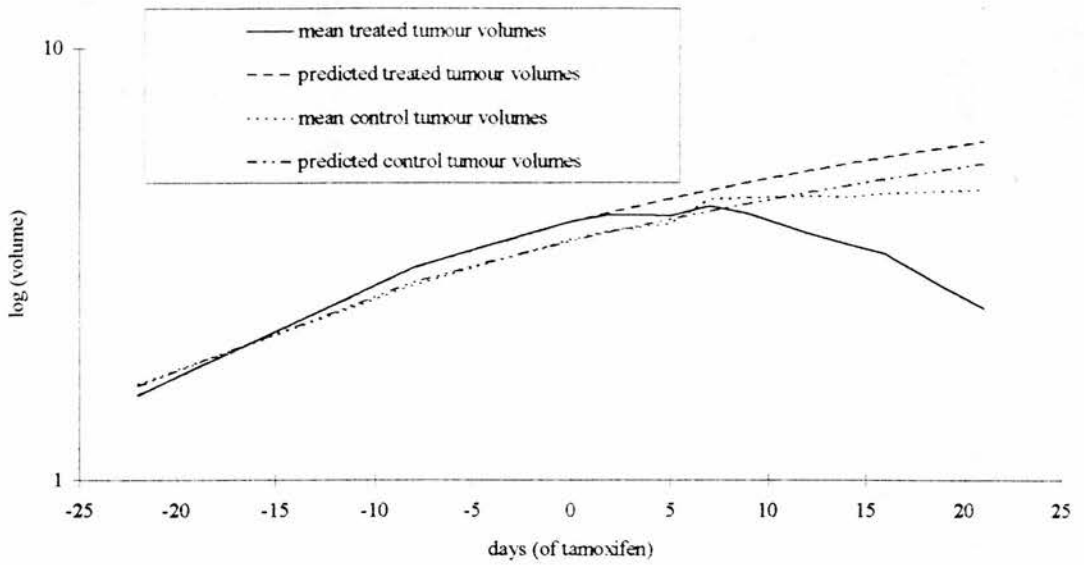


Figure 7.5.1 Predicted and actual mean volumes in ZR-75-1 xenografts subjected to sequential FNAs

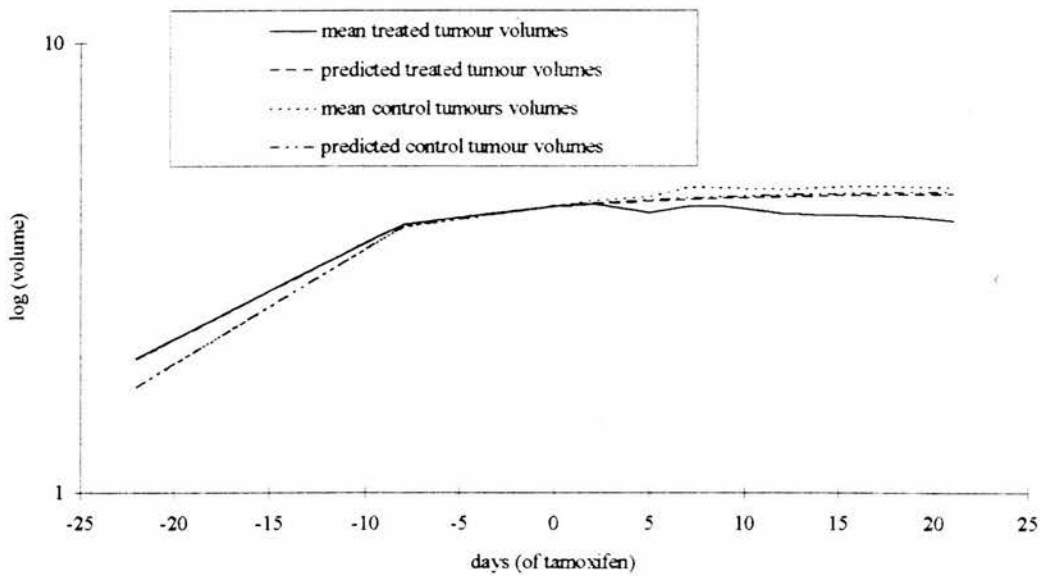


Figure 7.5.2 Predicted and actual mean volumes in MDA-231 xenografts subjected to sequential FNAs

However close examination of the curves suggests that the treated MDA-MB-231 tumours do not grow as fast as the control. There are in fact only five tumours under consideration, and three regressed; one after day 9 and two from day 12. The growth curves of these tumours is shown in figure 7.5.3 where it is clear that the pattern of growth regression is quite different from that seen in the ZR-75-1 tumours, in that it appears to begin before the tamoxifen therapy was commenced!

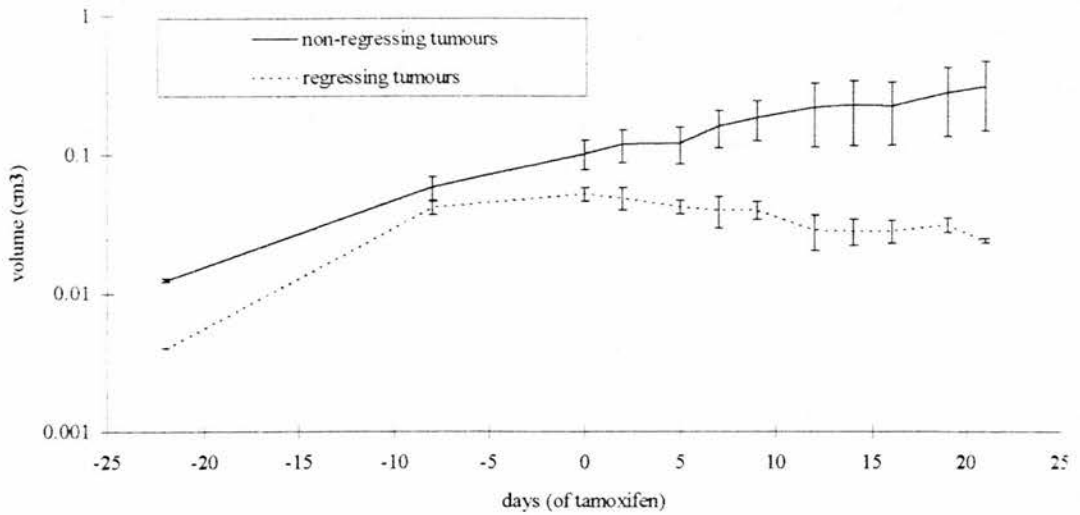


Figure 7.5.3 Growth curves for tamoxifen treated MDA-MB-231 tumours

These changes in growth are confirmed by the student-t test for the actual and predicted volumes, as shown in table 7.5.1, which confirms not only that the tamoxifen treated ZR-75-1 xenografts regressed, as assessed by the difference between the actual and predicted volumes, but that there was also a change in growth for the treated MDA-MB-231 tumours.

| volumes | ZR-75-1 | | MDA-MB-231 | |
|--------------|-----------|---------|------------|---------|
| | treated | control | treated | control |
| up to day 0 | n.s. | n.s. | n.s. | n.s. |
| beyond day 0 | < 0.00001 | n.s. | <0.01 | n.s. |
| all volumes | < 0.0002 | n.s. | <0.01 | n.s. |

Table 7.5.1 Student t-test comparisons between actual and predicted volumes for MDA-MB-231 and ZR-75-1 xenografts subjected to sequential FNAs.

There are few examples in the literature of the use of a function to fit and thus predict the subsequent growth of xenografted human cancers. The first study to do this reported no measures of goodness of fit, but pictorially showed that three early volume measurements could be sufficient to predict the subsequent (unperturbed) growth of a rat carcinoma (Norton et al(1976)). The only other study, which included a serially passaged human breast cancer, fitted curves to individual xenografts, but with a common, average, maximum tumour size, and thus reduced the growth variation permitted to an individual tumour (Spang-Thomsen et al(1980)). No goodness-of fit data were therefore available.

The critical issue for modelling tumour growth is not the degree of fit of the chosen function (Gompertz in this study), as when there are only three or four data points most bounded non-linear growth functions can be made to fit, but whether or not it is an accurate predictor of subsequent growth, assuming that there are no fundamental changes in the determinants of growth. Hence one cannot test the assumption that growth is truly Gompertzian in the treated tumours, and as has already been noted, there are problems with the ZR-75-1 control tumours removed on day 28, in that 4/10 regressed, including all three in the second passage (no. 29). Thus the accuracy of growth curve prediction will be assessed before day 28, and since the key changes in apoptosis, mitosis and their correlation with response occurred between days 2 - 14, it is over the first two weeks' of therapy that the predictive ability of the model is paramount.

There are two hypotheses that need to be tested. Firstly that any differences observed between predicted and observed control volumes at a certain time point are no more than would be expected by chance; and secondly that any differences between predicted and actual volumes will be significantly more in the treated ZR-75-1 tumours, as compared with control tumours (confirming tamoxifen induced growth regression), whereas for the MDA-MB-231 tumours growth prediction should be as accurate in control as treated tumours.

Section 7.1 confirms that although the Gompertz growth curve does not predict the precise volumes of the untreated ZR-75-1 xenografts, there are no significant differences between the actual and predicted volumes for the control tumours at any

point between day 0 and 28, using all volumes up to any time points therein. Furthermore, table 7.1.3 indicates that the prediction is accurate for up to 7 days beyond the last time point, although thereafter the residuals rise, suggesting that the lack of a statistical difference between the actual and predicted volumes could be due to the relatively small number of control tumours.

For the MDA-MB-231 tumours no such changes were seen, and there is in general much better growth prediction for the control tumours as well as the treated tumours (see section 7.2); which further confirms the observation that tamoxifen had no effect on the growth of these tumours. As commented upon there is only one time-point (day 14) where one group of treated tumours (those excised on day 28) had actual volumes that were significantly different from those predicted by the Gompertz function. This could have occurred by chance, although the lower confidence limit of 1% was used to reduce the possibility of such a type I error. There is indeed no other obvious explanation for this, as all the volumes measured on day 14 were not significantly different from those predicted by the individual growth curves.

The next group of tumours that had their growth modelled were from the experiment where different xenografts were grown in the opposite flanks of the same animal. As has already been commented upon (see section 7.4), there were fewer tumours and measurements of tumour volume available than had been intended. No differences were seen between the predicted and actual volumes for any group of tumours, but the small numbers prohibit any useful conclusion being drawn from this data. However they are consistent with the observations from the larger, earlier experiments. This experiment also afforded the possibility of examining the effect of growing different tumours in the same animal ; and for the few animals where this was possible, there were no differences in the V_{max} , a , or b for the two different tumours. This is consistent with the data of Brunton (Brunton et al(1978)) who noted that the Gompertz growth parameters were species, not tumour specific. However when considering the "average" Gompertz growth curves, as in tables 7.4.3 & 7.5.1, there is a suggestion that V_{max} is tumour-dependent, being smaller for the MDA-MB-231 tumours than the ZR-75-1 xenografts.

The growth of the tumours that were subjected to sequential FNAs needs to be considered. It is clear from section 7.5 that in the control tumours of both types, there was no evidence that sequential FNAs had any impact upon tumour growth. The treated ZR-75-1 tumours had a significant change in growth, confirmed by comparisons

of the treated volumes with both those seen in the untreated ZR-75-1 tumours, as well as those predicted by the growth curves. However the treated MDA-MB-231 tumours, as a group, appear to have less growth than was predicted, even if their volumes are not significantly different from the control MDA-MB-231 tumours at any time point. Figure 7.5.3 suggests however that the regression that occurred in three of the five tumours that survived beyond day 5, could be due to a change in growth that commenced before the tamoxifen treatment. No definite explanation can therefore be given, and the notion that these tumours responded to tamoxifen is inconsistent with all the prior data. However the data are equally inconsistent with the possibility of the sequential FNAs inducing significant alterations in growth, and thus the data on apoptosis & proliferation during this experiment can be considered as a reflection of the effects of time and/or tamoxifen in these tumours.

In conclusion therefore, the tumour volumes do appear to be well-modelled by the Gompertz function. The growth curves of the untreated tumours of both types can be predicted by the volumes measured up to day 0 ; however the confidence of the fit recedes with time, especially for the ZR-75-1 tumours. The volumes of the treated ZR-75-1 tumours are only different from the predicted volumes at day 7 and beyond ; whereas those of the treated MDA-MB-231 are not different for the duration of the experiment. The data indeed suggest that the use of a Gompertz curve to predict growth provides a more sensitive measure of differences in growth than a comparison of the volumes of treated and untreated tumours.

Discussion

Tamoxifen treatment of breast cancer is clearly associated with considerable clinical benefit, whether used to treat macroscopic, or as in the adjuvant setting, microscopic disease. A considerable body of data already exist as to the biological consequences of tamoxifen treatment of breast cancer, and the data presented here confirm some of these prior findings. In particular the anti-proliferative action of tamoxifen has been confirmed in both clinical tumours and xenografts. Although no reduction in mitotic activity was observed in the ER-negative MDA-MB-231 tumours, the data from the clinical breast cancers do not suggest that this anti-proliferative action is synonymous with clinical efficacy, in that it was observed in both responding and non-responding tumours, consistent with prior reports (Clarke et al(1993)). Furthermore tamoxifen treatment clearly increased the level of apoptosis in the sensitive ER-positive ZR-75-1, but not the insensitive MDA-MB-231 xenografts. Although the precise pattern of this change over time remains unclear, the analysis of both the histological sections and the FNAs strongly suggest that this increase is temporary, abating within two weeks' therapy. In contrast the clinical breast cancers when re-examined after three months' treatment showed no overall change in the incidence of apoptosis, which further implies that any increase in apoptosis induced by tamoxifen is transient, in contrast to the permanent reduction observed in proliferation.

The exact relationship between apoptosis and bcl-2 remains unclear for breast cancer. Undoubtedly the bcl-2 expressing clinical tumours have lower rates of apoptosis, but as already discussed, the rate of apoptosis also correlates with the rate of proliferation, and higher proliferative fractions are in general seen in ER and/or bcl-2 negative tumours. Such a direct relation between bcl-2 expression and lower proliferation was however not confirmed in the current series of ER-positive tumours. The available data do therefore suggest that bcl-2 expression may play at least some rôle in inhibiting apoptosis in clinical breast cancer. The effect of three months' tamoxifen was to reduce bcl-2 expression in the majority of tumours, and indeed the analysis in section 2.7 suggests that the tumours which respond to tamoxifen in the clinical sense of volume reduction, also do so biologically in that the fall in bcl-2 expression is accompanied by a rise in apoptosis at three months. However in the xenografts, those that respond clinically manifest no change in bcl-2 expression over the four weeks of the study, but a clear and probably transient rise in apoptosis occurs. Hence changes in apoptosis at least in the short term do not seem to necessitate alterations in bcl-2 protein levels, but longer-term reduction in bcl-2 can be associated with higher rates of apoptosis.

Although the results suggest some concordance between the data obtained from the analyses of the clinical breast tumours and the xenografts, there were in fact important differences in experimental design between these two studies. In particular, the timing of the acquisition of the on-treatment tumour samples was very different for the clinical and xenografted breast cancers. Tumour material was only available from the patients after three months' tamoxifen, whereas in the xenograft experiments all samples were taken within 28 days' treatment, with the most significant changes occurring in the first week. Nonetheless both studies show that there was a correlation between tumour response and respectively the change in the ratio and the absolute value of apoptotic:mitotic ratio. Furthermore the definitions of response were different - in the patients a comparison could only be made with the pre-treatment volume, whereas in the xenografts the use of a Gompertz growth curve allowed accurate prediction of how the tumours would have grown if left untreated, and thus the on-treatment volume could be contrasted with the predicted volume for the same time point. However table 7.3.3 confirms that there was also a significant correlation between the relative volume and the apoptotic:mitotic ratio, which corresponds more closely with the definition of response for the clinical tumours. These observations suggest that measuring the relative balance between tumour cell growth and death during tamoxifen treatment may allow identification of tamoxifen sensitivity. In the clinical breast cancers the relationship was in fact stronger between response and the pre-treatment level of ER expression and/or the change in ER expression. However in only two tumours did the level not fall with treatment, and all tumours were ER positive, so that the specificity of these changes to tamoxifen sensitivity remains unclear. The level of significance of the correlations between response to tamoxifen and the pre-treatment level of bcl-2 expression as well as the change in bcl-2 expression were similar to that found for the change in apoptotic:mitotic ratio. But the use of a sample taken after three months' treatment with which to compare the pre-treatment biology is potentially a less-specific measure of drug efficacy, as the subsequent behaviour of the individual tumour is more uncertain. In contrast, the identification of changes in apoptosis and proliferation during the first week of tamoxifen therapy in the xenografts, together with the observed tight correlation with response, suggests that such analyses do offer the potential for predicting for tamoxifen sensitivity.

Flow cytometry

The use of the cellular DNA content to estimate, using the flow-cytometer, the percentage of cells in different parts of the cell-cycle is a tried and tested technique. Given that the source material however is not from cell culture, but rather from FNAs, some thought needs to be given on the methodology, and its correlation with histopathological markers of proliferation. There is only one published study reporting proliferation in control and tamoxifen-treated breast cancer MCF-7 xenografts (Brunner et al(1989)), in which no significant changes in the percentages of cells in G₁, S or G₂/M were seen during 21 days' therapy. However in that same study, the volume reduction in the treated tumours was less than 50% after 21 days, considerably less than that presented here, but consistent with our own experience with xenografts of this cell line.

The data presented in section 3.7 suggests that in general the flow-cytometric and histopathological measures of proliferation are tightly correlated, with the exception of the percentage of cells in G₂/M and the mitotic index. This at first consideration appears unexpected, but the difference between these two variables is that the former contains cells in G₂. Given that an FNA from an untreated ZR-75-1 xenograft has on average 6% of its cells in this phase of the cell-cycle, whereas the average mitotic index in the same tumours is just under 0.1%, it is immediately clear that any small changes in the proportion of cells in mitosis will not be seen unless the percentage of cells in G₂ falls by the same degree. Furthermore it is now recognised that cells can undergo apoptosis at any point in the cell cycle (Kroemer et al(1995)), and therefore there may be varying numbers of cells in G₂ (identified as such by flow cytometer) which would subsequently be undergoing apoptosis and not mitosis.

The use of the fluoro-TUNEL technique was designed to give a reproducible measure of apoptosis in fine needle aspirates. However, as has already been commented upon in the introduction, DNA fragment detection systems cannot *per se* identify whether the DNA fragments are from (in) an apoptotic cell or a necrotic cell (Collins et al(1992); Gold et al(1994); Grasl-Kraupp et al(1995); Lizard et al(1995); Wijsman et al(1996)). When employed immunohistochemically, light microscopy can be used to aid the distinction (Wijsman et al(1996)), but this requires the use of at least some of the same criteria as used in a morphological assessment, and therefore does not totally avoid the problem of subjectivity. With the flow cytometer, no such visual assessments are practical. A small study was therefore done, with a cell sorter being used to separate the cells in the apoptotic and non-apoptotic regions. These groups of cells were then smeared onto a slide and examined

under fluorescent light. Morphologically apoptotic cells, identified by fluorescent, condensed blebbed chromatin were seen only in the cells from the apoptotic region (data not shown). This does not however confirm that all the apoptotic cells were in this region, nor that necrotic cells were not included. Hence the correlation between the histological and flow cytometric measures of cell death need to be critically examined. It has already been noted that whereas there is overall a correlation between the amount of apoptosis and necrosis, no such relationship exists between the FACS-measure of apoptosis and either measure of cell death alone. However although multivariate analysis did suggest that FACS-apoptosis was related to both, the only statistically significant factor was necrosis, with a low R^2 value. Hence one must interpret the results of this technique with a certain degree of caution.

Removal of necrotic (and late stage apoptotic) cells from the sample with a pre-treatment, such as that using DNAase (Darzynkiewicz et al(1984)), is possible. The data reported in section 6.2.2 confirm that the results correlate well with the fluoro-TUNEL method without pre-treatment. However it was not possible to re-examine all the FNAs using this technique, because there is the considerable risk that a further freeze-thaw cycle might increase the permeability of the cell membranes, which is the property of dead cells upon which the method relies to identify necrotic (and late stage apoptotic) cells.

Hence although the data from the flow cytometric analyses remain consistent with those obtained by histological examination of the sections, they are in general less sensitive to the changes observed with tamoxifen therapy. It remains unclear as to exactly why this is, but suggests that the use of FNAs of xenografts does not necessarily offer a solution to the difficulty of repeat biological examination from individual xenografts and possibly clinical tumours.

Conclusion

This study has focused upon changes that are consequent upon the tamoxifen treatment of breast cancer. The clinical data suggest that, as previously reported, the level of expression of ER and bcl-2 predict for tamoxifen-sensitivity. However the changes occurring after three months' therapy suggest that reduced expression of bcl-2 is only associated with a response when apoptosis is increased, yet the pre-treatment level of apoptosis does not predict for sensitivity. There are tentative data to suggest that tumours with a low initial apoptotic:mitotic ratio, and/or tumours whose ratio increase during therapy are more likely to be responders, and that these changes do not appear to be as closely related to alterations in bcl-2 expression as might at first be expected.

The model system considered xenografts of two well-established breast cancer cell lines, one hormone-sensitive (ZR-75-1) and the other hormone-insensitive (MDA-MB-231). Despite the variations in tumour growth, and in particular that of the untreated tumours, there are clear data confirming that only the former tumour is hormone-sensitive. Furthermore histological assessment confirms that tamoxifen sensitivity is associated with an increase in apoptosis and a fall in proliferation, best evidenced by the apoptotic:mitotic ratio. Using Gompertzian growth curves, fitted to individual tumours, one can identify tamoxifen-induced changes in the growth of the ZR-75-1 xenografts after only 7 days' therapy, at which point there is an extremely tight correlation with the apoptotic:mitotic ratio, suggesting that the relative balance between proliferation and apoptosis is the prime determinant of the change in growth in these tumours. In contrast, the other mechanism of cell loss, necrosis, would appear to have no part to play in tamoxifen induced tumour regression, at least in this model system. Furthermore tamoxifen treatment of the sensitive ZR-75-1 xenografts, but not the insensitive MDA-MB-231 tumours, clearly reduced the extent of tumour vasculature. However these changes coincided with the increase in apoptosis, and therefore it remains unclear as to which of these two processes is the prime determinant of regression, although the existence of a significant correlation between the degree of regression (response) and the apoptotic:mitotic ratio but not the vascular portion of the tumour favour the hypothesis that the change in vasculature is the consequence of rather than the cause of the tumour regression.

Attempts to circumvent the need for a repeat biopsy to establish the presence of these early changes in clinical tissue by the use of flow cytometry was not totally successful, in part because of the difficulty with the methodology for assessing apoptosis. However when several sequential samples were taken from the same tumour, the assay was more sensitive to tamoxifen-induced changes, but not sufficiently to predict whether or not the individual tumour would respond to therapy.

Clearly further work is required to validate the specificity of these changes as predicting for tamoxifen sensitivity - both in this model system, by using other cell lines, such as paired cell lines in which tamoxifen resistance has been induced in an initially sensitive line (such as ZR-75-1 and its resistant counterpart ZR-75/LCC3 (Kristensen et al(1995a))), and in the clinic. For the latter, further clinical studies need to be performed, using tumour material made available after 7-14 days' tamoxifen therapy. Such protocols are underway in the Edinburgh Breast Unit, with the intention of assessing changes in proliferation, apoptosis, angiogenesis as well as the neo-angiogenic growth factors such as VEGF (Ferrar et al(1989)) and basic FGF with which it may act in synergy (Goto et al(1993); Pepper et al(1992)). The relative timing of these changes and the specificity of their relationship to clinical tamoxifen sensitivity will then be determined.

However more fundamental questions are raised by this study. The exact mechanism by which tamoxifen induces apoptosis remain to be elucidated. No changes in bcl-2 or p53 were observed in the model system, but this does not exclude the possibility that the techniques used were insensitive to any change that might have occurred, nor does it exclude the involvement of other members of the bcl-2 family. Equally in the clinical study, the changes in bcl-2 expression and apoptosis confirm an inverse relationship in responding tumours, but whether they are causally linked or independent effects of a more fundamental change in tumour biology remains unclear.

Finally, one must consider briefly the implications of the correlation between proliferation and apoptosis. Tamoxifen clearly acts as an anti-proliferative agent, and in general the relation between apoptosis and proliferation is preserved, even if altered, during tamoxifen therapy. Hence if continuous tamoxifen exposure reduces the proliferative fraction of a tumour, with a concomitant reduction in apoptosis, this potentially undermines some of the initial cytotoxicity of the drug. It begs the question as to whether or not continuous or intermittent therapy might have a more significant anti-tumour action, as has been partially addressed by studies comparing sequential tamoxifen and progestogen therapy with continuous tamoxifen (Crawford et al(1992)).

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Without the advice and assistance of E. Miller I would have not have succeeded in the immunohistological and flow cytometric analyses of the material collected.

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